IMPERIAL AGRICULTURAL
RESEARCH INSTITUTE, NEW DELHI.

CONTENTS OF VOLUME 128

No. 1, April, 1939

PAGE
Reineke, E. P., Peterson, V. E., and Turner, C. W. The partition of the serum globulins of the dairy goat 1
Goettsch, Marianne, Lonstein, Ida, and Hutchinson, John J. Muscle phosphorus in nutritional muscular dystrophy in rabbits .. 9
Frost, D. V., and Elvehjem, C. A. Factor W and its relation to the vitamin B complex 23
Jukes, Thomas H. Experiments with the filtrate factor 35
McHenry, E. W., and Gavin, Gertrude. The B vitamins and fat metabolism. II. The effect of thiamine upon the synthesis of body fat in pigeons 45
Ball, Eric G. Xanthine oxidase: purification and properties 51
Poo, L. J., Lew, W., and Addis, T. Protein anabolism of organs and tissues during pregnancy and lactation 69
Bernheim, Frederick, and Bernheim, Mary L. C. Note on the action of manganese and some other metals on the oxidation of certain substances by liver 79
Fevold, H. L. Chemical differences of the follicle-stimulating and luteinizing hormones of the pituitary 83
Hess, W. C., and Sullivan, M. X. The cystine content of deaminized proteins 93
Orten, James M., and Smith, Arthur H. On the site of the formation of citric acid in the animal organism 101
Cohn, David J., Tannenbaum, Albert, Thalhimer, William, and Hastings, A. Baird. Influence of oxygen and carbon dioxide on the blood of normal and pneumonic dogs 109
Eichelberger, Lillian. The distribution of body water in skeletal muscle in dogs with impaired renal function 137
Flock, Eunice V., Hester, Harold R., and Bollman, Jesse L. Phospholipid changes during the production of fatty livers in geese 153
Schneider, H., and Steenbock, H. A low phosphorus diet and the response of rats to vitamin D_2 159
Gray, J. S., Ivy, A. C., and Cuthbert, F. P. The conversion of protein to glucose in depancreatized and phlorhizinized dogs 173
Block, Richard J. The composition of keratins. The amino acid composition of hair, wool, horn, and other eukeratins 181
Embree, Norris D. The occurrence of cyclized vitamin A in fish liver oils 187

Stekol, Jakob A. Studies on the mercapturic acid synthesis in animals. XI. The detoxication of benzyl chloride, benzyl alcohol, benzaldehyde, and S-benzylhomocysteine in the rabbit and rat 199

Kies, Marian Wood, Dyer, Helen M., Wood, John L., and du Vigneaud, Vincent. A study of the utilization of the optical isomers of N,N′-dimethylcystine 207

Bergmann, Max, and Stein, William H. A new principle for the determination of amino acids, and its application to collagen and gelatin 217

Greenstein, Jesse P Sulfhydryl groups in proteins. II. Edestin, excelsin, and globin in solutions of guanidine hydrochloride, urea, and their derivatives 233

Greenstein, Jesse P. Studies of multivalent amino acids and peptides. XI. The synthesis of diglycyl-*l*-cystine 241

Greenstein, Jesse P., and Klemperer, Friedrich W. Aspartylhistidine 245

Horecker, B. L., Stotz, Elmer, and Hogness, T. R. The promoting effect of aluminum, chromium, and the rare earths in the succinic dehydrogenase-cytochrome system 251

Eck, John C , and Thomas, Byron H. The chemical activation of sterols. V A study of the relationship between chemical activation and configuration of various sterols and derivatives 257

Eck, John C., and Thomas, Byron H. The chemical activation of sterols. VI. A study of various reagents in chemical activation and in sterol color reactions 267

Ratish, Herman D., Bullowa, Jesse G. M., Ames, James B., and Scudi, John V. Urinary excretion products of sulfanilyl-2-aminopyridine 279

Winter, Irwin C. Fat metabolism following liver injury. Decreased iodine number of tissue fatty acids of male rats following carbon tetrachloride administration 283

Bernhart, F. W. Denaturation and molecular splitting of egg albumin by ultraviolet radiant energy 289

Fenn, W. O. The deposition of potassium and phosphate with glycogen in rat livers 297

Wilde, W. S. The distribution of potassium in the cat after intravascular injection 309

Meyer, Karl, Smyth, Elizabeth M , and Dawson, Martin H. The isolation of a mucopolysaccharide from synovial fluid .. 319

Roe, Joseph H., and Hall, James M. The vitamin C content of human urine and its determination through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid 329

Beach, Eliot F., Erickson, Betty Nims, Bernstein, Samuel S., Williams, Harold H., and Macy, Icie G. The amino acid composition of erythrocyte posthemolytic residue of five mammalian species 339

NEURATH, HANS, and SAUM, ARTHUR M. The denaturation of serum albumin. Diffusion and viscosity measurements of serum albumin in the presence of urea.......... 347
GUERRANT, RALPH E., and HOGAN, ALBERT G. Effect of amino acids on anemia caused by deaminized casein.......... 363
TREADWELL, C. R., and ECKSTEIN, H. C. The composition of the tissue lipids of the Virginia white-tailed deer (*Odocoileus virginianus borealis*).......... 373
ADLERSBERG, DAVID, and ELLENBERG, MAX. Effect of carbohydrate and fat in the diet on uric acid excretion.......... 379

No. 2, MAY, 1939

MONTGOMERY, M. L., ENTENMAN, C., and CHAIKOFF, I. L. The liver lipids of dogs subjected to ligation of the external pancreatic ducts 387
TOENNIES, GERRIT, and KOLB, JOSEPH J. Methionine studies. II. *dl*-Methionine sulfoxide.......... 399
TWEEDY, WILBUR R., TEMPLETON, R. D., PATRAS, MARY C., McJUNKIN, F. A., and McNAMARA, E. W. Studies on the effects of calciferol in the thyroparathyroidectomized-nephrectomized rat.. 407
MAIN, EDNA R., and SHINN, LAWRANCE E. The determination of hydrogen peroxide in bacterial cultures.......... 417
CARR, C. JELLEFF, and FORMAN, SYLVAN E. Sugar alcohols. XX. The fate of *d*-sorbitol, styracitol, and *l*-sorbose in the animal body 425
JACOBS, WALTER A., and CRAIG, LYMAN C. Delphinine. II. On oxodelphinine.......... 431
JACOBS, WALTER A., ELDERFIELD, ROBERT C., and CRAIG, LYMAN C. The aconite alkaloids. II. The formula of oxonitine.......... 439
GREEN, LOWELL F., McCARTHY, JAMES F., and KING, C. G. Inhibition of respiration and photosynthesis in *Chlorella pyrenoidosa* by organic compounds that inhibit copper catalysis.......... 447
McCARTHY, JAMES F., GREEN, LOWELL F., and KING, C. G. The substrate specificity and inhibition characteristics of two copper-protein "oxidases".......... 455
BAER, ERICH, and FISCHER, HERMANN O. L. Studies on acetone-glyceraldehyde. IV. Preparation of $d(+)$-acetone glycerol...... 463
BAER, ERICH, and FISCHER, HERMANN O. L. Studies on acetone-glyceraldehyde. V. Synthesis of optically active glycerides from $d(+)$-acetone glycerol.......... 475
BAER, ERICH, and FISCHER, HERMANN O. L. Studies on acetone-glyceraldehyde. VI. Synthesis of the biological $l(-)$-α-glycerophosphoric acid.......... 491
KOHLER, G. O., ELVEHJEM, C. A., and HART, E. B. The relation of pyrrole-containing pigments to hemoglobin synthesis.......... 501
WEBB, T. J., and KNIAZUK, MICHAEL. Spectrophotometric examination of blood from animals receiving sulfanilamide.......... 511

GURIN, SAMUEL, BACHMAN, CARL, and WILSON, D. WRIGHT. The gonadotropic hormone of urine of pregnancy. I. A simple method of extraction and purification.... 525
BRATTON, A. CALVIN, and MARSHALL, E. K., JR. A new coupling component for sulfanilamide determination.... 537
EDER, HOWARD, BRADLEY, H. C., and BELFER, SAMUEL. The survival of cathepsin in autolysis.... 551
GOERNER, ALFRED, and GOERNER, M. MARGARET. Vitamin A and liver cell tumors.... 559
MCCARTHY, EARL F., and VAN SLYKE, DONALD D. Diurnal variations of hemoglobin in the blood of normal men.... 567
HOWELL, STACEY F. The determination of the urea in chicken blood.. 573
CHARGAFF, ERWIN. Unstable isotopes. I. The determination of radioactive isotopes in organic material 579
CHARGAFF, ERWIN. Unstable isotopes. II. The relative speed of formation of lecithin and cephalin in the body 587
ROBERTS, RICHARD G. Some reactions of ammonolyzed insulin.... 597
RITTENBERG, D., SCHOENHEIMER, RUDOLF, and KESTON, ALBERT S. Studies in protein metabolism. IX. The utilization of ammonia by normal rats on a stock diet.... 603
TIPSON, R. STUART, CHRISTMAN, CLARENCE C., and LEVENE, P. A. The structure of the aldobionic acid from flaxseed mucilage.... 609
JELLINEK, E. MORTON, and LOONEY, JOSEPH M. Statistics of some biochemical variables on healthy men in the age range of twenty to forty-five years.... 621
JONES, H. B., CHAIKOFF, I. L., and LAWRENCE, JOHN H. Radioactive phosphorus as an indicator of phospholipid metabolism. VI. The phospholipid metabolism of neoplastic tissues (mammary carcinoma, lymphoma, lymphosarcoma, Sarcoma 180) 631
LONGENECKER, HERBERT E. Deposition and utilization of fatty acids. I. Fat synthesis from high carbohydrate and high protein diets in fasted rats.... 645
KLEIN, J. R. Effect of thyroid feeding and thyroidectomy on the oxidation of amino acids by rat kidney and liver.... 659
VIRTUE, ROBERT W., and DOSTER-VIRTUE, MILDRED E. Studies on the production of taurocholic acid in the dog. IV. Cysteine, homocysteine, and thioglycolic acid.... 665

NO. 3, JUNE, 1939

JOSEPH, MICHAEL, COHN, WALDO E., and GREENBERG, DAVID M. Studies in mineral metabolism with the aid of artificial radioactive isotopes. II. Absorption, distribution, and excretion of potassium 673
VICKERY, HUBERT BRADFORD, and PUCHER, GEORGE W. The loss of carbon from excised rhubarb leaves during culture.... 685
VICKERY, HUBERT BRADFORD, and PUCHER, GEORGE W. The metabolism of amides in green plants. III. The mechanism of amide synthesis.... 703

JACOBS, WALTER A., and CRAIG, LYMAN C. The ergot alkaloids. XVII. The dimethylindole from dihydrolysergic acid.......... 715
KNUDSON, ARTHUR, STURGES, STUART, and BRYAN, W. RAY. Cholesterol content of skin, blood, and tumor tissue in rats irradiated with ultraviolet light.......... 721
WYCKOFF, RALPH W. G. The ultracentrifugal analysis of the latent mosaic virus protein.......... 729
PERLMAN, I., and CHAIKOFF, I. L. Radioactive phosphorus as an indicator of phospholipid metabolism. VII. The influence of cholesterol upon phospholipid turnover in the liver.......... 735
NORDBÖ, R. The concentration of ionized magnesium and calcium in milk.......... 745
LANGSTROTH, G. O., and TALBOT, N. B. The absorption spectra of the compounds formed by androsterone and testosterone in the *m*-dinitrobenzene reaction.......... 759
PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS..... iii
INDEX TO VOLUME 128.......... 775

THE PARTITION OF THE SERUM GLOBULINS OF THE DAIRY GOAT*

BY E. P. REINEKE, V. E. PETERSON, AND C. W. TURNER

(From the Department of Dairy Husbandry, University of Missouri, Columbia)

(Received for publication, November 23, 1938)

During the course of investigations in progress in this laboratory on the blood precursors of milk constituents, there was need for a method of determining accurately the different fractions of the serum globulin complex of the dairy goat. Reports in the literature do not agree as to the number of globulins that are present in blood plasma or serum, nor the extent to which they can be separately determined in routine analysis.

In the method of Howe (1, 2) a euglobulin and two pseudoglobulin fractions are determined by precipitation with sodium sulfate. Campbell and Hanna (3) published a similar method for human serum, using sodium sulfite as the precipitant. By the use of increasing concentrations of methyl alcohol, Liu and Wu (4) obtained breaks in precipitation of the serum proteins of the dog, ox, horse, and sheep corresponding to euglobulin, pseudoglobulin, and albumin. Butler and Montgomery (5) and Roche *et al.* (6) reported breaks in the solubility curves of horse sera that indicated the separation of euglobulin and one pseudoglobulin. In the case of human serum, however, they state that no convincing evidence of a break in solubility was obtained over the entire globulin range.

In addition to a "globoglycoid" separated from crystalline albumin, Hewitt (7) announced the separation of three main types of globulin fractions, including two euglobulins and one pseudoglobulin. Each of these globulins was divided further into three fractions of different carbohydrate content. Tiselius (8) presented evidence of three globulins in horse serum, differing in their electrophoretic properties. Stenhagen (9) reported corresponding results for human serum. Supporting evidence for the existence of three globulin fractions in the serum of cattle is given by Howe (10) who reported that the blood of new born calves remains extremely low in its content of euglobulin and pseudoglobulin I until after colostrum is ingested.

---

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series, No. 586.

Whether or not there is actually a difference in the number of globulins contained in the sera of different species, it appears that globulin fractions can be prepared which differ considerably in their solubility patterns, and in the extent to which they may be partitioned in single precipitations by the salting-out technique. Thus it seemed questionable whether methods of fractionation evolved for the determination of the plasma or serum proteins of other species would apply in the case of the goat. The experiments reported in this paper were conducted in order to gain some information on this point.

EXPERIMENTAL

Blood samples were drawn from the abdominal mammary vein or carotid artery of goats, in flasks coated with potassium oxalate, and were centrifuged immediately to remove the plasma. The globulins were precipitated from separate portions of plasma by the use of a graded series of sodium sulfate solutions, ranging in concentration from 14 to 24 per cent and differing by intervals of 1 per cent. Since high dilution of the samples has been shown to effect a more complete separation of fractions (1, 3, 11, 12), all precipitations were carried out at a dilution of 1:31.

15 ml. of sodium sulfate solution were added to duplicate 0.5 ml. portions of oxalated plasma. The plasma sulfate samples were placed in an incubator at 38° and allowed to remain, with occasional shaking, for at least 3 hours or until precipitation was complete. The samples were filtered twice at 38° through Whatman No. 50 filter paper. Total nitrogen was determined on the filtrates by the official Kjeldahl method.

The results are plotted as the mg. of nitrogen remaining in the filtrate per 100 cc. of plasma, against the per cent of sodium sulfate in the solution; and also as the log of the solubility against increasing salt concentration. According to Cohn's equation

(13) the log of the solubility of the protein plotted against the concentration of salt should form a succession of straight lines of different slope. The intersection of two of these lines marks the end of the precipitation of one protein and the beginning of precipitation of another.

Fig. 1 shows the solubility curves obtained on goat plasmas subjected to a graded series of sodium sulfate solutions, differing in concentration by 1 per cent. Breaks in solubility are indicated at 15 to 16 per cent, 18 to 19 per cent, and 22 to 23 per cent of sodium sulfate. The fact that there is some additional precipitation of globulins between 22 and 23 per cent of sodium sulfate, but none between 23 and 24 per cent, indicates that the critical zone for total globulins must lie between salt concentrations of 22 and 23 per cent. Although breaks between three globulin fractions are indicated, there is no point in the globulin range where the solubility of a particular fraction reached zero, indicating complete separation.

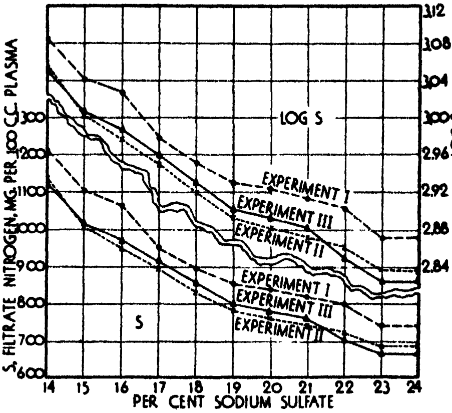


Fig. 1. Solubility of the globulins of goat plasma in increasing concentrations of sodium sulfate solution. Concentration interval = 1.0 per cent sodium sulfate.

A study of the curves depicted in Fig. 1 led us to believe that by narrowing the concentration intervals of the salting-out solutions to 0.5 per cent one should be able to establish more definitely the critical zones of precipitation that are indicated. Therefore, a new series of solutions was prepared, differing in concentration by intervals of 0.5 per cent and covering the range of 14 to 23 per cent sodium sulfate. At the same time the size of the sample used for each determination was increased to 1.0 ml. of plasma and 30 ml. of sodium sulfate solution. Analyses were made of three additional plasma samples and two serum samples, all drawn at different times, and representing five different goats.

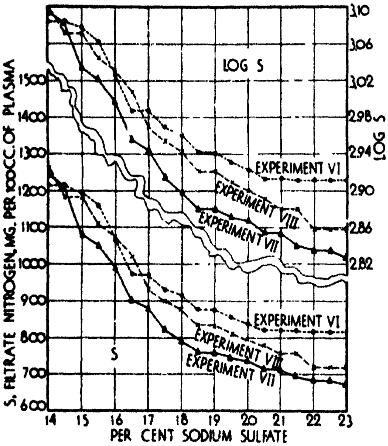


FIG. 2

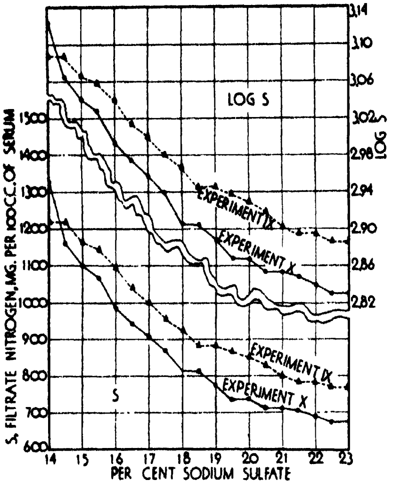


FIG. 3

FIG. 2. Solubility of the globulins of goat plasma in increasing concentrations of sodium sulfate solution. Concentration interval = 0.5 per cent sodium sulfate.

FIG. 3. Solubility of the serum globulins of the goat in increasing concentrations of sodium sulfate solution. Concentration interval = 0.5 per cent sodium sulfate.

Experiments VI to VIII, Fig. 2, were run on plasma, while Experiments IX and X, Fig. 3, represent analyses of serum.

As might be expected, there are variations in the solubility curves of different samples representing different goats. However, inspection of the curves will show that there are definite breaks, or critical zones of precipitation, corresponding in general to the points established for bovine serum by Howe.

Experiment VI, Fig. 2, and Experiment IX, Fig. 3, show an initial break in the curve from 14.0 to 14.5 per cent of sodium sulfate. Since the analysis was made on serum in Experiment IX, no fibrin was present, and it is apparent that the first globulin fraction started to precipitate with a sodium sulfate concentration in excess of 14.5 per cent.

Experiments VI and VIII show distinct breaks in the solubility curves at concentrations of 15.0 and 14.5 per cent of sodium sulfate, respectively. The remaining curves also show indications of a break in precipitation at 14.5 to 15.5 per cent, but they leave something to be desired in the way of sharpness. Apparently there is considerable overlapping of precipitation limits in most goat plasmas between the two fractions separated partially at this point.

A uniform and distinct break appears in all of the solubility curves at 15.5 per cent of sodium sulfate. This coincides closely with the critical zone between pseudoglobulin I and II established by Howe for cattle serum.

The globulin complex precipitated by a concentration of sodium sulfate in the range from 18.5 to 22.5 per cent is quite variable in its response. Although the increments of protein precipitated in each case are small and might possibly be attributed to experimental error, there are in most of the curves, notably Experiments VII to X, indications of two additional globulin fractions instead of the one (pseudoglobulin II) previously reported. There are also indications of a break at 16.5 to 17.0 per cent of sodium sulfate, which would represent the partial subdivision of pseudoglobulin I into two fractions with overlapping limits of solubility.

All samples analyzed showed a distinct break in precipitation with 22.5 per cent sodium sulfate, indicating that the salting-out of globulins is complete at this point.

From the evidence presented it would appear that the concentrations of sodium sulfate solution most nearly representing the critical zones for the determination of the three recognized fractions of the serum globulins of the goat are as follows: euglobulin, 15 per cent; pseudoglobulin I, 18.5 per cent; pseudoglobulin II, 22.5 per cent. In all of these experiments the serum proteins were precipitated at a dilution of 1:31. Therefore, the above percentages correspond to a final sulfate concentration in the plasma sulfate solutions of 14.51, 17.90, and 21.77 per cent, respectively.

In Table I a comparison is given between Howe's (2) revised sodium sulfate concentrations and those arrived at in this laboratory for goat plasma.

The sodium sulfate concentrations selected are 0.2 to 0.3 per cent higher in each case than those of Howe when expressed in terms of percentage concentration, or 0.02 to 0.03 mole higher when expressed in terms of molarity.

Howe (14) reported that after the first fraction has been precipitated the additional amount of salt required for a subsequent fraction is a constant for a given salt. He found the increment of salt for sodium sulfate to be 0.25 mole. Our experimentally determined points differ by 0.24 and 0.27 mole, respectively. Inasmuch as the solutions were made in terms of percentage concentration instead of molarity, this can be considered as quite good agreement. The critical zones for different plasmas apparently vary over a range of approximately 0.5 to 1 per cent of sodium sulfate.

TABLE I

Critical Precipitation Points of Goat Plasma As Compared to Cattle Plasma

Fraction	Bovine plasma (Howe (2))			Goat plasma (this paper)		
	Final concentration	Molar concentration	Increment of salt	Na_2SO_4, final concentration	Molar concentration	Increment of salt
	per cent		*mole*	*per cent*		*mole*
Fibrinogen	10.6	0.75				
Euglobulin	14.2	1.00	0.25	14.51	1.021	
Pseudoglobulin I	17.7	1.25	0.25	17.90	1.260	0.24
" II	21.5	1.50	0.25	21.77	1.532	0.27

SUMMARY

Recent findings in regard to the separation of the serum globulins are reviewed briefly, with particular reference to differences reported in the partition of the globulins of various species.

Solubility curves are presented, showing the changes in solubility of the serum globulins of the goat, with increasing sodium sulfate concentration.

From breaks in the solubility curves, it is concluded that the serum globulins of the goat can be partitioned into the globulin fractions euglobulin, pseudoglobulin I, and pseudoglobulin II, established by Howe for bovine serum. These fractions can be salted-out at a dilution of 1:31 with sodium sulfate solutions of 15.0, 18.5, and 22.5 per cent, respectively.

By narrowing the interval between sulfate concentrations, additional breaks in solubility were obtained at sodium sulfate concentrations of 16.5 to 17.0 per cent and 20.0 to 21 per cent. These are considered to represent subdivisions of pseudoglobulin I and pseudoglobulin II.

BIBLIOGRAPHY

1. Howe, P. E., *J. Biol. Chem.*, **49**, 93 (1921).
2. Howe, P. E., *J. Biol. Chem.*, **57**, 235 (1923).
3. Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, **119**, 9 (1937).
4. Liu, S.-C., and Wu, H., *Chinese J. Physiol.*, **8**, 97 (1934).
5. Butler, A. M., and Montgomery, H., *J. Biol. Chem.*, **99**, 173 (1932-33).
6. Roche, A., Darier, M., and Samuel, L., *Compt. rend. Soc. biol.*, **121**, 1019 (1936).
7. Hewitt, L. F., *Biochem. J.*, **32**, 26 (1938).
8. Tiselius, A., *Biochem. J.*, **31**, 1464 (1937).
9. Stenhagen, E., *Biochem. J.*, **32**, 714 (1938).
10. Howe, P. E., *J. Biol. Chem.*, **49**, 115 (1921).
11. Haslam, H. C., *J. Physiol.*, **32**, 267 (1905).
12. Gille, R., *Compt. rend. Soc. biol.*, **121**, 906 (1936).
13. Cohn, E. J., *Physiol. Rev.*, **5**, 349 (1925).
14. Howe, P. E., *J. Biol. Chem.*, **57**, 241 (1923).

MUSCLE PHOSPHORUS IN NUTRITIONAL MUSCULAR DYSTROPHY IN RABBITS*

By MARIANNE GOETTSCH, IDA LONSTEIN, AND JOHN J. HUTCHINSON

(From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, December 27, 1938)

Rabbits and guinea pigs upon certain diets develop an extreme degeneration of the skeletal muscles (15, 22). The onset of these muscle lesions may be delayed or prevented by the inclusion of vegetable oils in the diet (21, 26, 31) or by vitamin E concentrates (23). In dystrophic muscle the creatine content is lower than normal and roughly proportional to the amount of pathological degeneration (14, 27); there is an increase in moisture and a decrease in total nitrogen (14), an increase in the rate of oxygen consumption (38, 21), a decrease in glycogen, total acid-soluble phosphorus, and fractions thereof, and an increase in cholesterol content (27), a gain in sodium chloride and a corresponding loss in potassium and magnesium, and an increase in calcium (10, 25).

In order to describe dystrophic muscle more completely, analyses of total phospholipid, total acid-soluble phosphorus, and total inorganic orthophosphate phosphorus were made upon the muscle of about 50 normal rabbits and the same number of rabbits in various stages of nutritional muscular dystrophy. Determinations of phosphocreatine and inorganic orthophosphate phosphorus in resting muscle were carried out in a few animals. The values obtained were compared with the extent of pathological degeneration and creatine content.

EXPERIMENTAL

The muscles for analysis were obtained from young and adult rabbits on Diets 11 or 13 (15) with or without supplements of

---

* This work was aided by the Research Grant from The Chemical Foundation, Inc., to this Department and by a grant from the Rockefeller Foundation.

natural foods or vegetable oils, or on a diet of grains (10). Normal controls were kept on a stock diet of grains, alfalfa hay, and greens. The rabbit was usually killed by a blow and immediately sampled, although in some instances material was taken some hours after death. For determinations on resting muscle, the rabbit was anesthetized with sodium amytal (Lilly), and the muscle frozen *in situ* with a mixture of carbon dioxide snow and ethyl chloride, cut in fine slices or powdered in a cold mortar, and the trichloroacetic acid extract prepared according to the method of Davenport and Davenport (8).

The muscles were analyzed separately. The gluteus was used for most of the determinations, but occasionally the gastrocnemius and triceps brachii (caput longum) were also taken. The muscle was freed as far as possible from connective tissue and each pair sampled for chemical analysis and histological study.

Methods

For moisture, the samples were dried for 20 hours at 100°. Creatine was determined by the method of Rose, Helmer, and Chanutin (35). The phosphorus was measured colorimetrically by the Fiske and Subbarow (13) method. For total phosphorus, the dried muscle was incinerated with magnesium nitrate; for phospholipid phosphorus, an alcohol-ether extract of the fresh tissue cut in small pieces was made; for total acid-soluble and total inorganic orthophosphate phosphorus, the finely cut or frozen and powdered muscle was extracted with 5 per cent trichloroacetic acid, the acid being allowed to remain in contact with the tissue for at least 30 minutes. Phosphocreatine was determined by the indirect method of Fiske and Subbarow (13) on extracts prepared by the method of Davenport and Davenport (8).

There was an average difference of 4 per cent between twenty-four pairs of duplicate determinations of total phosphorus in muscle.

Results

The results of the analyses of the muscle phosphorus fractions in normal and degenerated muscle are shown in Figs. 1 to 3 and

Tables I and II. The muscle was arbitrarily graded from 1 + to 4 +, according to the severity of the pathological lesions.[1]

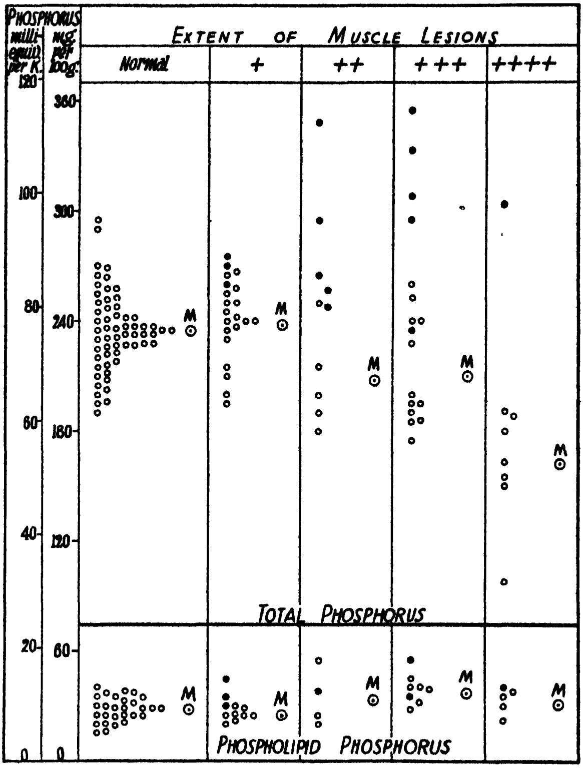


FIG. 1. Concentration of total and phospholipid phosphorus in white muscle of normal rabbits and those with nutritional muscular dystrophy. Circles represent total and phospholipid phosphorus; solid dots, determinations of calcified muscles. *M* shows the position of the mean value (excluding determinations of calcified muscles).

Total Phosphorus—Total phosphorus is reported to remain constant during muscle contraction (7) and atrophy (17). A marked reduction has been observed in the muscle of rabbits, in which acidosis was induced by feeding hydrochloric acid (16).

---

[1] We are indebted to Dr. A. M. Pappenheimer of the Department of Pathology, Columbia University, for his interpretation of the pathology of the muscles.

The results of analyses of total phosphorus in normal and degenerated muscle are plotted in Fig. 1. As the phosphorus content of normal muscle appeared to have no relation to age, within the limits of 2 months to 2 years, the records of all rabbits have been tabulated together regardless of age.

The following figures, calculated from the analytical values for total phosphorus, are expressed as milliequivalents per kilo of fresh tissue, on the assumption that the phosphorus is present as a monobasic acid: 59 determinations, mean 75.8 ± 0.6, standard deviation ±7.0 ± 0.4, coefficient of variation 9.2 ± 0.6. The coefficient of variation is about the same as that for creatine and total nitrogen (14). The mean is slightly lower than the values obtained with the Neumann gravimetric method for rabbit muscle by Katz (19), Goto (16), and Sorg (37), but higher than those of Morgulis and Osheroff (25).

In Fig. 1 it is seen that in dystrophic muscle the total phosphorus fluctuates widely. It may increase to a value higher than that indicated in Fig. 1; five severely degenerated muscles with a total phosphorus concentration of from 137 to 322 milliequivalents were not included. This increase has been shown to be related to the amount of calcification in the fibers (10). In dystrophic muscle without calcification the total phosphorus varies widely and tends to decrease. In muscle with slight or moderate lesions the total phosphorus may remain normal or decrease to some extent, but in severely degenerated muscle the decrease is significant. These results are not in agreement with those of Morgulis and Osheroff (25), who found no alteration in the total phosphorus of severely dystrophic muscles, but their series may not have included muscle with as great pathological degeneration as those classified as 4 + in Fig. 1.

Phospholipid Phosphorus—Phospholipid phosphorus is stated not to change during contraction (7) or atrophy (17). A slight decrease has been observed during fasting (6).

The results of analyses of phospholipid phosphorus in normal and dystrophic muscle are plotted in Fig. 1. The concentration of phospholipid phosphorus in normal muscle is seen to vary greatly. The calculated values for normal muscle are, for twenty-eight determinations, mean 9.1 ± 0.3 milliequivalents per kilo of fresh muscle, standard deviation ±2.1 ± 0.2, coefficient of variation 23.6. The method used for extracting phospholipids tends to give high values (36). The values obtained are in general agreement with those given by Katz (19) and Bloor and Snider (1), and somewhat lower than those of Sorg (37).

Although there were relatively few determinations of phospholipid phosphorus, it appears from Fig. 1 that this fraction differs, in degenerated muscle, but little from the normal. This finding, which is contrary to that of Morgulis, Wilder, Spencer, and Eppstein (28) who stated that the phospholipid phosphorus is increased in dystrophic muscle, is of particular interest, because the fat content of such muscle may be great. For example, the muscle of Rabbit 51 presented 4 + lesions; the gluteus contained 73 per cent fat by analysis and the triceps, 25 per cent; the phospholipid phosphorus content was respectively 7.1 and 9.7 milliequivalents per kilo.

The presence of calcified fibers in dystrophic muscle does not affect the phospholipid phosphorus.

Total Acid-Soluble Phosphorus—Total acid-soluble phosphorus remains constant during contraction (7), autolysis (4), fasting (39), fever induced by the injection of 1,2,4-dinitrophenol (5), and in some types of human muscle disorder, such as myasthenia gravis, Graves' disease, and periodic muscular weakness (29). Low values have been found in thyroxinized and adrenalectomized cats (4), in rats following denervation (18), in human muscle diseases, such as familial periodic paralysis (2), muscle dystrophy (33), pseudohypertrophic muscular dystrophy and dystrophia myotonica (29), and in nutritional muscular dystrophy of the rabbit (27).

Fig. 2 illustrates the data on total acid-soluble phosphorus content of normal and dystrophic muscle. The concentration of total acid-soluble phosphorus in normal muscle is seen to be more variable than that of total phosphorus, a fact which may be due to the method of extraction. The values calculated for normal tissue are, for forty-four determinations, mean 50.2 ± 0.9 milliequivalents per kilo of fresh tissue, standard deviation ±9.8 ± 0.6, coefficient of variation 19.4. These values are in agreement with those given in the literature (24, 4, 29).

In degenerated muscle the total acid-soluble phosphorus varies more than in normal ones. Higher concentrations are found in calcified tissue, in which it may increase to 3 times the normal amount. The analytical values for four severely degenerated muscles, in which the concentration of total acid-soluble phosphorus varied from 117 to 153 milliequivalents, were not plotted.

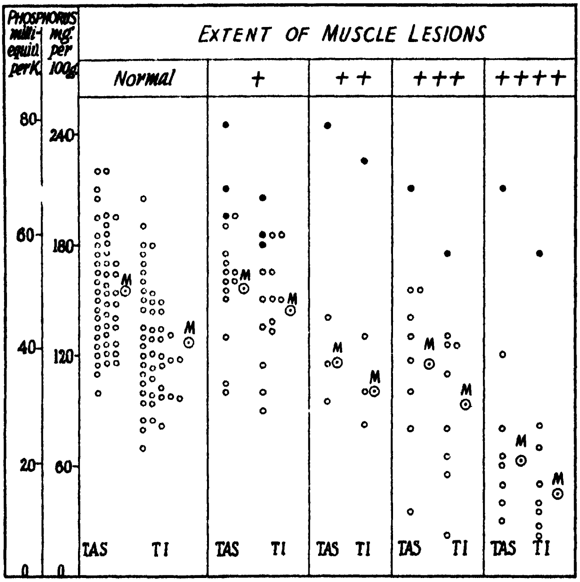


FIG. 2. Concentration of total acid-soluble (*TAS*) and total inorganic orthophosphate (*TI*) phosphorus in white muscle of normal rabbits and those with nutritional muscular dystrophy. Circles represent total acid-soluble and total inorganic orthophosphate phosphorus; solid dots, determinations of calcified muscles. *M* shows the position of the mean value (excluding determinations of calcified muscles).

It is seen in Fig. 2 that lower concentrations are found in uncalcified dystrophic muscle and that the decrease is significant in severely degenerated muscle. This observation confirms that of Morgulis and Spencer (27) and is in agreement with the lowered total acid-soluble phosphorus found by Brand and Harris (2), Nevin (29), and Reinhold *et al.* (33, 34) in human muscle diseases.

Total Inorganic Orthophosphate Phosphorus—Inorganic orthophosphate phosphorus increases rapidly during muscle contraction (20) and autolysis, and is derived chiefly from the hydrolysis of phosphocreatine (12, 9).

The amount of total inorganic orthophosphate phosphorus present in normal and dystrophic muscle is also shown in Fig. 2. The following values were calculated: forty-four determinations, mean 42.4 ± 1.0 milliequivalents per kilo of fresh tissue, standard deviation ±9.4 ± 0.7, coefficient of variation 22.2.

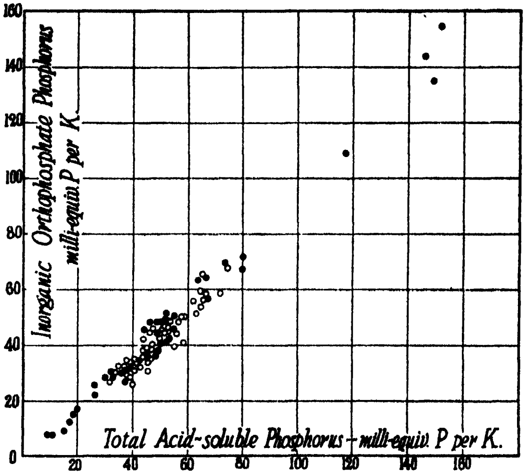


FIG. 3. Relationship between total acid-soluble and total inorganic orthophosphate phosphorus in white muscle of normal rabbits and those with nutritional muscular dystrophy. Circles represent determinations of normal muscle; solid dots, of muscle with lesions.

Fig. 3 presents a scatter diagram of the concentration of total acid-soluble phosphorus and total inorganic orthophosphate phosphorus, showing their relationship in all types of muscle. Total inorganic orthophosphate phosphorus is roughly 85 per cent of the total acid-soluble phosphorus in postmortem muscle, irrespective of its pathological state.

Phosphocreatine and Inorganic Orthophosphate Phosphorus in Resting Muscle—Phosphocreatine phosphorus is low in the resting muscles of guinea pigs with scurvy and pigeons with polyneuritis (30), in fasting (39), in rats following denervation (18), in thyroxinized and adrenalectomized cats (4), in fever induced by the injection of 1,2,4-dinitrophenol (5), in molecular degeneration of muscle in rabbits, produced by freezing or contusion (11), in human muscle dystrophies (29, 34), and in nutritional muscular dystrophy of the rabbit (27).

The concentrations of phosphocreatine and inorganic orthophosphate in resting muscle were determined in seven rabbits with muscular dystrophy, four controls, and one rabbit with extreme muscular atrophy.

The results are given in Table I, together with analyses of other constituents. Whenever possible the analyses are given in duplicate in order to show the variation between samples, the extent of which is not surprising when the heterogeneous histological picture of the muscle is considered.

The values for phosphocreatine in normal resting muscle are similar to those given in the literature (24, 4, 29). In dystrophic muscle with advanced lesions the phosphocreatine phosphorus of resting muscle decreases to very low values, comparable to those found by Nevin (29) in human muscular dystrophy and lower than those reported by Morgulis and Spencer (27) in the rabbit disease. However, although the amount of phosphocreatine is less in degenerated muscle, there is apparently little decrease in the relative concentration. Thus, it will be seen in Table I that the phosphocreatine content of normal muscle is 35 to 40 per cent of the total acid-soluble fraction. In dystrophic muscle with 3 + and 4 + uncalcified lesions, the proportion of phosphocreatine decreases slightly, but not more than might be accounted for by analytical error. In calcified muscle, Rabbits 351 and 368, the phosphocreatine appears to be slightly higher than in the uncalcified dystrophic muscle.

In normal resting muscle the inorganic orthophosphate phosphorus content is one-third to one-half as great as that of phosphocreatine phosphorus, but in degenerated muscle the proportion is greater. In all cases, regardless of the state of the muscle, the sum of these two fractions is approximately equal to 60 per cent of the total acid-soluble phosphorus.

Phosphorus Fractions in Muscle Atrophy—The analytical results of Rabbit 366, which presented extreme muscular atrophy, are included in Table I. It will be seen that the moisture is increased, creatine is less than normal, and although the fractions of phosphorus are low, they are within the limits of biological variation of normal muscle. The results are in agreement with those of Grund (17).

TABLE I

Phosphorus Fractions in Normal and Pathological Rabbit Gluteus Muscle

			Pathological examination of muscle				Phosphorus, m. eq. per kilo fresh tissue						
							Post mortem				Resting muscle		
	Rabbit No.	Days on diet	Severity of lesions	Extent of calcification	Per cent H_2O	Creatine, m. eq. per kilo fresh tissue	Total	Phospholipid	Total acid-soluble	Total inorganic orthophosphate	Total acid-soluble	Phosphocreatine	Inorganic orthophosphate
Normal diet	61		–	–	76.1	37.4	75.8		58.4		52.3	17.8	8.1
											51.0	18.7	8.4
	62		–	–		38.9	83.4	9.3	54.7		49.6	20.0	7.3
											42.2	18.2	6.0
	63		–	–	76.9	32.0	78.7		44.2		40.3	15.1	15.1
											42.6	14.7	16.8
	64		–	–	75.4	35.9	72.9		45.1		55.5	22.2	14.6
Grain diet and modifications	366*	87	–	–	80.6	27.5	58.1	9.8	42.0	33.2	36.7	14.7	4.5
							59.1	10.3	42.6	29.5	41.5	15.7	10.5
	319	156	+	–		44.3	74.6	8.9	53.4	48.5	42.5	13.2	16.0
	351	107	+	+	75.9	45.8	91.5	14.3	66.0	57.8	60.9	15.5	25.4
	363	134	+++	–		22.9	61.9	12.6	38.3	30.3	35.5	9.8	11.6
	368	138	+++	++	76.7	32.0	98.8	17.8	68.0	56.9	71.6	12.0	29.8
Diet 11 and modifications	110	166	+++	–	80.0	13.0	55.8		24.8		10.0	3.3	6.6
											13.5	3.4	6.7
	29†	314	++++	–		16.0	50.0		15.5		16.1	4.0	5.1
	78‡	161	++++	–		9.1	48.4		20.3		21.3	5.4	9.7

The muscles had normal fat content, except as indicated. For additional analytical data on some of these muscles see Goettsch and Brown (14) and Victor (38).

* Muscle presented extreme atrophy.

† Muscle fat, 5.3 per cent.

‡ Muscle fat, 20.6 per cent.

Acid-Insoluble Non-Lipid Phosphorus—On comparison of Figs. 1 and 2 it will be seen that the concentration of total acid-soluble phosphorus in normal rabbit muscle is significantly lower than that of total phosphorus. There is a difference of 25.6 milliequivalents per kilo of fresh tissue between the two means, with a standard deviation of the difference of ±12.0. This acid-insoluble fraction was determined in several muscles by ashing the residue of the trichloroacetic acid extraction. The analyses of a typical muscle (normal gluteus of Rabbit 267) were as follows: total phosphorus 76.8 milliequivalents per kilo of fresh muscle, total acid-soluble phosphorus 47.8, 51.9, total acid-insoluble phosphorus 27.6, 25.2, sum of acid-soluble and acid-insoluble phosphorus 75.4, 77.1.

When the residue of the alcohol-ether extraction of the same muscle was ashed and analyzed, the phosphorus content was found to be higher than that of total acid-soluble phosphorus. The analyses of the normal gluteus of Rabbit 267 were as follows: phospholipid phosphorus 6.5, 7.7 milliequivalents per kilo of fresh muscle, non-phospholipid phosphorus 66.4, 64.1, sum of phospholipid and non-phospholipid phosphorus 72.9, 71.8. The difference between the non-phospholipid phosphorus and the total acid-soluble phosphorus, about 15 milliequivalents, represents the acid-insoluble non-lipid phosphorus, which is presumably nuclear and protein phosphorus. Similar differences between total and total acid-soluble phosphorus in normal dog muscle were reported by Pollack, Flock, and Bollman (32).

From Figs. 1 and 2 it appears that the total acid-soluble phosphorus decreases at a greater rate than the total phosphorus. Since the phospholipid phosphorus remains constant, this observation leads to the assumption of an increase in acid-insoluble non-lipid phosphorus in dystrophic muscle, which is compatible with the histological finding of increased nuclear elements. The increase may also be accounted for by an error in analysis, due to the extraction of fibrous lipomatous tissue with water-soluble reagents. The latter explanation was found to be the more likely.

Powdered frozen muscle was extracted first with alcohol-ether, and then with several portions of trichloroacetic acid. The residue was ashed and analyzed for phosphorus. The analyses of typical muscles are given in Table II. The residues of the dystrophic muscles contained approximately the same amount of phosphorus as the normal ones.

Relationship of Phosphorus Fractions and Creatine Nitrogen—Scatter diagrams indicated little correlation between the phosphorus fractions and creatine nitrogen. The coefficients of correlation with their respective probable errors, calculated from forty-four sets of determinations of normal and dystrophic muscle, are as follows: total phosphorus and creatine nitrogen +0.038, total acid-soluble phosphorus and creatine nitrogen +0.552 ± 0.07, total inorganic orthophosphate phosphorus and creatine nitrogen +0.506 ± 0.07. These results are not in accordance with the statement of Brown and Imrie (3) that there is a tendency for the concentration of total acid-soluble phosphorus to be high in cat muscle when the creatine is high. Their value for total acid-soluble phosphorus represents total inorganic orthophosphate phosphorus, since they measured the amount of phosphate in the trichloroacetic acid extract after 50 minutes hydrolysis. When the coefficient of correlation was calculated from their data, it was found to be +0.440 ± 0.10, a value in general agreement with the one given above.

TABLE II

Acid-Insoluble Non-Lipid Phosphorus in Normal and Pathological Rabbit Gluteus Muscle

Rabbit No.	Pathological examination of muscle	Phosphorus, m. eq. per kilo fresh tissue				
		Total	Phospholipid	Total acid-soluble	Residue	Lipid + acid-soluble + residue
345	Normal	78.6	11.8	57.3	8.5	77.6
368	+++	98.8	17.8	68.0	8.9	94.7
382	+++	64.4	12.6	32.5	16.3	61.4
384	+++	82.0	13.8	50.1	11.9	75.8

From Table I it is apparent that no clear correlation exists between creatine and the other fractions of phosphorus.

In nutritional muscular dystrophy of the rabbit the loss in muscle creatine is greater in equivalent amount than the loss in phosphorus. Furthermore, the decrease in creatine content is apparent in muscles with slight or moderate lesions, while the changes in phosphorus fractions are not regularly observed until the muscles are severely degenerated. The phosphorus concentration is more strikingly increased than that of creatine in muscles showing histological evidence of calcification.

SUMMARY

In nutritional muscular dystrophy of the rabbit, there are no striking changes in the muscle phosphorus fractions until the muscles are severely degenerated. Dystrophic muscles which present calcification histologically are associated with an increase in total, total acid-soluble, and total inorganic orthophosphate phosphorus; those without calcified fibers, with a decrease in these constituents.

The phosphocreatine content of resting degenerated muscles is distinctly lower than normal, but its relationship to total acid-soluble phosphorus remains the same.

There is no change in phospholipid phosphorus of muscle.

BIBLIOGRAPHY

1. Bloor, W. R., and Snider, R. H., *J. Biol. Chem.*, **107**, 459 (1934).
2. Brand, E., and Harris, M. M., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **97**, p. lxii (1932).
3. Brown, M., and Imrie, C. G., *J. Physiol.*, **71**, 214 (1931).
4. Buell, M. V., Strauss, M. B., and Andrus, E. C., *J. Biol. Chem.*, **98**, 645 (1932).
5. Cahn, T., *Ann. physiol. et physicochim. biol.*, **9**, 393 (1933).
6. Cahn, T., and Bonot, A., *Ann. physiol. et physicochim. biol.*, **4**, 485 (1928).
7. Cuthbertson, D. P., *Biochem. J.*, **19**, 896 (1925).
8. Davenport, H. A., and Davenport, H. K., *J. Biol. Chem.*, **76**, 651 (1928).
9. Eggleton, P., and Eggleton, G. P., *J. Physiol.*, **63**, 155 (1927).
10. Fenn, W. O., and Goettsch, M., *J. Biol. Chem.*, **120**, 41 (1937).
11. Fishback, D. K., and Fishback, H. R., *Arch. Path.*, **14**, 204 (1932).
12. Fiske, C. H., and Subbarow, Y., *Science*, **65**, 401 (1927).
13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
14. Goettsch, M., and Brown, E. F., *J. Biol. Chem.*, **97**, 549 (1932).
15. Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.*, **54**, 145 (1931).
16. Goto, K., *J. Biol. Chem.*, **36**, 355 (1918).
17. Grund, G., *Arch. exp. Path. u. Pharmakol.*, **67**, 393 (1912).
18. Hines, H. M., and Knowlton, G. C., *Am. J. Physiol.*, **104**, 379 (1933).
19. Katz, J., *Arch. ges. Physiol.*, **63**, 1 (1896).
20. Macleod, J. J. R., *Z. physiol. Chem.*, **28**, 535 (1899).
21. Madsen, L. L., *J. Nutrition*, **11**, 471 (1936).
22. Madsen, L. L., McCay, C. M., and Maynard, L. A., *Cornell Univ., Agric. Exp. Stat., Mem. 178* (1935).
23. Mattill, H. A., *Proc. XVI Internat. Physiol. Cong.*, Zurich, 112 (1938).
24. Milroy, T. H., *Physiol. Rev.*, **11**, 515 (1931).
25. Morgulis, S., and Osheroff, W., *J. Biol. Chem.*, **124**, 767 (1938).
26. Morgulis, S., and Spencer, H. C., *J. Nutrition*, **11**, 573 (1936).

27. Morgulis, S., and Spencer, H. C., *J. Nutrition*, **12**, 173 (1936).
28. Morgulis, S., Wilder, V. M., Spencer, H. C., and Eppstein, S. H., *J. Biol. Chem.*, **124**, 755 (1938).
29. Nevin, S., *Brain*, **57**, 239 (1934).
30. Palladin, A., and Epelbaum, S., *Biochem. Z.*, **204**, 140 (1929).
31. Pappenheimer, A. M., and Goettsch, M., *Proc. Soc. Exp. Biol. and Med.*, **34**, 522 (1936).
32. Pollack, H., Flock, E., and Bollman, J. L., *Am. J. Physiol.*, **110**, 105 (1934–35).
33. Reinold, J. G., Clark, J. H., Kingsley, G. R., Custer, R. P., and McConnell, J. W., *J. Am. Med. Assn.*, **102**, 261 (1934).
34. Reinold, J. G., and Kingsley, G. R., *J. Clin. Inv.*, **17**; 377 (1938).
35. Rose, W. C., Helmer, O. M., and Chanutin, A., *J. Biol. Chem.*, **75**, 543 (1927).
36. Sinclair, R. G., *Physiol. Rev.*, **14**, 351 (1934).
37. Sorg, K., *Z. physiol. Chem.*, **182**, 97 (1929).
38. Victor, J., *Am. J. Physiol.*, **108**, 229 (1934).
39. Wesselkina, W. M., *Z. ges. exp. Med.*, **85**, 463 (1932).

FACTOR W AND ITS RELATION TO THE VITAMIN B COMPLEX*

By D. V. FROST† and C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, December 19, 1938)

Several conditions tend to complicate investigations on the newer factors in the vitamin B complex. Foremost of these is the species difference in the requirement of recognized factors. Witness for example the large and easily demonstrated requirement of the dog and pig for nicotinic acid as opposed to the apparently slight and difficultly demonstrated requirement of the rat and chick for this compound. Demonstration of the existence of water-soluble factors other than vitamin B_1, riboflavin, and vitamin B_6 essential to optimum growth of rats has appeared in the last 2 years from several laboratories (1–4). Variations in source materials, in the methods of fractionation, and in assay methods used in the different laboratories make conjecture as to the identity of these independently discovered factors difficult. A brief statement of unreported properties of Factor W, which may be helpful in relating it to other little known members of the vitamin B complex, therefore seems timely.

In a previous paper (2) we described Rations K_{17}, K_{19}, and K_{20}, all of which have been used in assay of Factor W preparations. Ration K_{20}, which has dextrin as the sole source of carbohydrate, was used for most of the assays reported herein. It has the following composition: dextrin 74, casein (Labco) 18, Salts I (5) 4, cottonseed oil 2, cod liver oil 2, vitamin B_1 (Merck) 200 micrograms, riboflavin (synthetic) 300 micrograms. Male rats placed on the ration at 21 to 25 days of age grow slowly to a constant weight range of 60 to 80 gm. Piebald rats maintained on the ration for 18 weeks have shown no noticeable graying of black areas and no clear cut or constantly recurring deficiencies of any description, except complete growth failure. All animals retain the appearance of normal young rats until they are about 100 days old, when they may exhibit some slight muscle dystrophy as evidenced by spastic gait. Administration of Factor W preparations to depleted animals of various ages has resulted uniformly in large growth responses ranging up to 55 gm. in 1 week. Rats maintained more than 14 weeks on the basal ration do not recover so completely or rapidly as do younger animals. For assay purposes rats of 7 to 10 weeks of age were used.

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

† Eli Lilly and Company Fellow.

Crystalline vitamin B_6, supplied to us by Dr. Samuel Lepkovsky, fed at a level of 40 micrograms per day did not stimulate growth of depleted animals. Since typical vitamin B_6 deficiency has been obtained on rations similar to Ration K_{20}, except that sucrose or starch was used as the source of carbohydrate in place of dextrin, a sparing action may be attributed to dextrin for at least partial requirement of vitamin B_6 in rats. Morgan, Cook, and Davison (6) have shown the sparing action of lactose for the vitamin B_6 requirement in rats and have attributed its action to intestinal synthesis which is sufficient to meet the normal requirement of the vitamin. Whether or not a partial deficiency of vitamin B_6 may be overcome by addition of Factor W concentrates at times to give greater growth than that attributable to the Factor W addition alone, is not known. Certainly vitamin B_6 is not the limiting factor for growth on Ration K_{20}, but its addition to the ration in crystalline form is a desirable one.

Methods

The procedure for obtaining preparations of Factor W for study of its properties has been previously reported (2) and is briefly as follows: acid acetone extracts of 95 per cent alcohol-soluble liver extract (Wilson[1]) were treated with a single batch of fullers' earth (Sargent) at pH 1 to 3 to remove the riboflavin. The filtrate contained considerable vitamin B_6 and nicotinic acid, as shown by assay with rats and dogs, and had full growth-promoting properties of liver extracts, as measured on our ration with growing rats. Fractionation of the fullers' earth filtrate with mercuric acetate had been shown to effect a separation of factors having slight supplementary growth-promoting properties. A similar separation of factors was possible with barium hydroxide in 75 per cent ethyl alcohol. The filtrate in each case promoted by far the greater growth stimulation for young rats and subsequently described properties may apply only to the factor, or factors, contained in such filtrates. The rate of growth over a 4 to 5 week period is the single criterion of activity of fractions and such assays are of insufficient duration for the animals to show any plateau in absence of the factor precipitated by mercury or barium.

---

[1] We are indebted to Dr. David Klein, The Wilson Laboratories, Chicago, for generous supplies of this material.

Precipitation Procedures—The organic acid-precipitating agents used included silver nitrate, copper acetate, lead acetate, and the previously mentioned mercuric acetate and barium hydroxide in 75 per cent ethyl alcohol. Organic base-precipitating agents included picric acid, picrolonic acid in butanol solution, mercuric chloride in 95 per cent ethyl alcohol, and chloroplatinic acid in dry ethyl alcohol. In all procedures the fullers' earth filtrate was used and when aqueous solutions were used the beginning concentration was approximately 1 cc., equivalent to 0.2 gm. of liver extract. The concentration was somewhat higher in those cases in which alcoholic solution was used. Precipitations were carried to completion. Measurements of pH were made on a Coleman electrometer and adjustments of pH were generally made with HCl or NaOH. In all cases most of the activity was recovered from the filtrate and in no case did the precipitate fraction carry appreciable activity, although some material was removed from solution by each precipitant.

Norit Adsorption and Elution—Adsorption of Factor W quantitatively from the fullers' earth filtrate with norit A (Pfanstiehl) required the use of about as much norit as there was solid material in solution. Successive adsorptions with small amounts of norit proved more efficient than a single treatment with a large amount. About equal results were obtained at pH 1 and 6. Completely decolorized solutions in many cases retained some activity, but in general the disappearance of activity from the solution paralleled the disappearance of color.

Successful elutions were made by refluxing the well dried norit with four successive portions of either dry butanol or a 1:1 mixture of benzene and absolute ethyl alcohol. 20 per cent suspensions of the norit in the solvent were used and the time of refluxing was 10 to 12 minutes. Aqueous pyridine-methanol mixture (2:1:1) used in much the same way proved equally effective in removing the active moiety. In all cases it was noted that the norit was completely stripped of adsorbed material before full recovery of activity was realized. Earlier attempts (2) to elute such norit preparations with aqueous pyridine-methanol were unsuccessful, but at that time the difficulty of elution was not appreciated and insufficient solvent was used. Also heat treatment, which now appears essential to successful elution of the factor from this type of norit, was not applied.

The norit adsorbate before elution was completely inactive for Factor W when fed to rats. Previously Elvehjem, Koehn, and Oleson (1) used a less powerful decolorizing carbon to adsorb the alcohol-ether precipitate factor, *i.e.* Factor W, and found the charcoal to be active for Factor W when fed.

Acetylation Procedure—When the fullers' earth filtrate fraction was dried, dissolved in a minimum of pyridine, and refluxed with an equal volume of acetic anhydride, the resulting mixture of water-soluble and water-insoluble materials was found to be quite inactive. Attempts to regenerate the activity from either the water-soluble or water-insoluble acetyl fractions by alkaline hydrolysis with ammoniacal methyl alcohol (4 N) at 100° were without success. Whether Factor W was destroyed by the acetylation or hydrolysis procedures, or whether the right conditions for hydrolyzing a physiologically inactive acetylated Factor W preparation were not used, is not known.

Extraction Procedures—When aqueous solutions of Factor W at pH 1, 6, and 9.5 were extracted six times with equal portions of butanol, the activity was removed in large part from the aqueous phase. When such butanol extracts were briefly shaken with an amount of 0.1 N NaOH just sufficient to maintain the pH of the solution at 12 to 14, all activity passed into the aqueous phase. When aqueous solutions of Factor W were extracted with butanol at pH 12 to 14, none of the activity was transferred to the butanol.

Phenol and aniline proved to be somewhat better solvents for removing Factor W from aqueous solution than did butanol;

Table I

Factor W Potency of Various Concentrates from Liver Extract

All experiments were conducted with the fullers' earth filtrate, fraction of comparable activity.

Experiment No.	Treatment	Fraction	Equivalent of original liver extract given daily	No. of rats	Wks. of experiment	Average wkly. increase in weight
			gm.			gm.
1	Acid acetone extraction of 95% EtOH-soluble liver	Extract	0.2	5	5	29
2	Fullers' earth adsorption of above, pH 1-3. 10% solution of fullers' earth	Filtrate	0.2	8	5	28.3
		Fullers' earth	0.4	2	5	2.2
3	Ether extraction (72 hr.), pH 1	Extract	0.4	4	5	10.8
		Residue	0.4	4	5	13.5
		Extract + residue	0.2 + 0.2	2	5	10
4	*n*-BuOH extraction, pH 1	Extract	0.3	2	5	17.3
		Residue	0.3	2	5	12.3
	" " " 6-7	Extract	0.4	2	5	27
		Residue	0.4	2	5	14
	" " " 9.5	Extract	0.4	2	4	24
		Residue	0.4	2	4	12.5
	" " " 14	Extract	0.3	2	4	0
		Residue	0.3	2	2	24.2
5	Phenol " " 0.3	Extract	0.3	4	4	20
		Residue	0.3	2	4	9.8
	" " " 6.7	Extract	0.3	3	4	23
		Residue	0.3	3	4	7.0
6	Norit elution with pyridine-MeOH-H_2O mixture (2:1:1)	Eluate	0.4	4	4	24
	Norit elution with dry *n*-BuOH	"	0.4	2	4	23.5
	Norit elution with benzene-absolute EtOH (1:1)	"	0.6	2	4	18.5
	Norit elution with absolute EtOH	"	0.5	2	4	9
7	Precipitation by $Hg(OAc)_2$, pH 8	Filtrate	0.4	4	4	16
		Ppt.	0.4	4	4	8.2
		Filtrate + ppt.	0.2 + 0.2	4	4	19.2
	Precipitation by $Pb(OAc)_2$, pH 8-9	Filtrate	0.4	2	5	21.5
		Ppt.	0.4	2	4	8
		Filtrate + ppt.	0.2 + 0.2	2	4	16
	Precipitation by H_2PtCl_6 in dry EtOH solution	Filtrate	0.4	1	5	20.4
	Precipitation by $HgCl_2$ in 95% EtOH solution	"	0.6	3	4	18.2

TABLE I—*Concluded*

Experiment No.	Treatment	Fraction	Equivalent of original liver extract given daily	No. of rats	Wks. of experiment	Average wkly. increase in weight
			gm.			*gm.*
8	Acetylation with Ac_2O in dry pyridine solution	H_2O-soluble	0.4	4	4	9.1
		H_2O-insoluble	0.4	2	3	4.8
9	Hydrolysis of H_2O-soluble acetyl with 2 N NH_3 in MeOH solution at 100°, 1 hr.	Hydrolysate	0.4	4	4	9.8

however, they also dissolved more solids than did butanol. Four extractions of a fullers' earth filtrate fraction, containing the equivalent of 40 gm. of liver extract, with 30 gm. portions of phenol sufficed to remove most of the activity from the water. This offers a convenient method for freeing the factor from most inorganic salts. Upon simultaneous extraction of phenol solutions of Factor W with water and ether, the activity passed to the water phase and the phenol was separated in the ether phase.

Continuous extraction of acid acetone extracts at pH 1 or 6 with ether over a period of 3 days did not prove useful as a concentration procedure. Neither the extracts nor the residues had the full activity of the original material, but all showed some portion of activity when fed alone. There appeared to be no separation of factors between the extract and residue, since combination of the two produced no greater growth than either alone. Animals fed the ether extract or residue from the procedure carried out at pH 1 showed extremely variable growth response; some animals responded fairly well for a considerable period and others declined and died following a good initial response during the first 2 or 3 weeks of supplement. Generation of toxic factors by the acid-ether treatment was thought to occur and may serve to becloud the interpretation of results. Results with the fractions made at pH 6 were essentially comparable to those made at pH 1, but were more uniform and no deaths occurred among the assay animals.

Typical results of most of the procedures reported are shown in Table I.

DISCUSSION

From the foregoing properties it is obvious that Factor W is not vitamin B_6, nicotinamide, nicotinic acid, the chick antidermatitis factor, or riboflavin. The extreme solubility of all preparations of Factor W in water and water-like solvents and practically complete insolubility in ether and such water-unlike solvents suggest that it is a compound, or mixture of compounds, having a high degree of hydroxylation. Difficulties inherent in separation of such types of compounds from mixtures of many water-soluble compounds appear to apply in attempts made thus far to separate Factor W in pure form.

Various investigators have reported the existence of growth factors in fullers' earth filtrates from yeast, liver, rice bran, wheat germ, and milk. That more than one factor affecting growth may be present in our filtrate from liver has already been shown (2) and it seems likely that a separation of factors may be accomplished with filtrates from other sources. The filtrate factor of Edgar and Macrae (4) from yeast appears to be similar to Factor W in many important respects. The growth obtained is comparable, failure to precipitate the factors with many common reagents is in agreement, and recently Macrae and Edgar (7) have reported extraction of a factor from deproteinized liver with amyl alcohol which they state may be identical with their yeast filtrate factor. The amyl alcohol fractionation used by Macrae and Edgar is comparable to the procedure developed by Elvehjem and Koehn (8) for concentration of the chick antidermatitis factor and to procedures described in this paper for concentration of Factor W. In our experience isoamyl alcohol is a better solvent for Factor W than *n*-amyl alcohol and about equal to *n*-butyl alcohol, which we have preferred to use.

Edgar and Macrae (4) concluded from results of barium hydroxide precipitation in 90 per cent ethyl alcohol of the fullers' earth filtrate factor prepared from yeast that the factor was carried down in the precipitate, but that in an identical precipitation made on a similar fraction prepared from liver the major part of the activity was not precipitated. In our experience a separation of fractions having supplementary activity was affected by precipitation of the fullers' earth filtrate from liver with barium hydroxide in 75 per cent ethyl alcohol solution, but the major portion of growth activity appeared in the filtrate.

The relation which Factor W bears to Factor 2 of Lepkovsky, Jukes, and Krause (3) is not clear, although growth has been reported for Factor 2 on rations combining all other known factors similar to that which Factor W preparations produce on our Ration K_{17} or K_{20}.

The criteria of activity of vitamin H of Booher (9) differ considerably from the criteria of activity established for Factor W and comparison is again difficult. Schultz and Mattill (10) have suggested the similarity of the filtrate factor prepared by them from heat-treated yeast to Factor W and also suggest its similarity to filtrate factors for the rat as prepared by other workers.

Exhaustive treatment with English fullers' earth according to the method of Lepkovsky, used in this laboratory by Oleson, Bird, Elvehjem, and Hart (11), apparently prevents recovery of Factor W, since no combination of filtrates or eluates allows the full qualitative effect of the original liver extracts. In our experience English fullers' earth is more destructive of Factor W than is Sargent's fullers' earth and is less efficient in removal of riboflavin at pH 1 to 3. Factor 2 of Lepkovsky, Jukes, and Krause, designated by them as the filtrate remaining after exhaustive extraction with fullers' earth, may not be identical with Factor W, as shown by Oleson, Bird, Elvehjem, and Hart (11).

The original alcohol-ether precipitation procedure of Elvehjem, Koehn, and Oleson (1) produced a clean separation of Factor W from the chick antidermatitis factor, since the precipitate was shown to have no curative or prophylactic properties of the chick factor, and the alcohol-ether filtrate contained little or no Factor W as measured with rats. Also, according to the method of Woolley, Waisman, Mickelsen, and Elvehjem (12), the chick factor is extractable from liver extracts with ether at pH 1. Factor W, as herein reported, is not extracted *in toto* by this procedure and the majority of it seems to remain in the residue.

Since active preparations of Factor W used in this work probably contain some nicotinic acid and nicotinamide and since the part played by these compounds in the nutrition of the rat is still mooted, we will briefly state our experience with them.

Following the work of Warburg and von Euler with the pyridine nucleotides and taking suggestion from the reports of Funk and von Euler that nicotinic acid and nicotinamide had slight salutary effect on the economy of rats deficient in the vitamin B complex, we had fed these compounds to rats maintained on our ration, K_{17}, designed for assay of Factor W (2). Both of these compounds exhibited growth-promoting action at that time. Our hypothesis that nicotinamide was important nutritionally as a precursor of the pyridine nucleotides was further supported by the fact that nicotinamide plus adenine nucleotide supported greater growth response than did either alone. Having exhausted the supply of adenylic acid with which growth stimulation was obtained, we resorted to Hoffmann-La Roche adenylic acid. This preparation elicited no response either alone or in combination with the pyridine compounds. When it became apparent that Factor W was not these compounds alone, we abandoned further trials with them for the time. The first effect obtained with adenine nucleotide may become explainable with further knowledge of Factor W and related growth factors. At this time, however, some clarification of the influence of the pyridine compounds on the growth of rats appears possible from comparison of results obtained elsewhere and in this laboratory.

Numerous investigators (7, 13–15) have reported failure of nicotinic acid and nicotinamide to effect the growth of young rats. In all these cases, rations based on sucrose and starch were used, supplemented generally with crystalline vitamin B_1 and riboflavin, and various combinations of fullers' earth eluate and filtrate fractions from liver, yeast, and rice bran, designed to supply rat antidermatitis and growth factors. In our earlier work (2) we obtained considerable growth stimulation in rats with both nicotinic acid and nicotinamide and it is important to note that the ration used was K_{17}, based on dextrin and containing 12 per cent of white corn. In subsequent work in which Ration K_{17} was used, these compounds have given stimulatory effects when fed with certain liver fractions. In later attempts to repeat growth effects with the pyridine compounds, Ration K_{20}, which contains no corn, was used. In all such cases there resulted consistent failure of nicotinamide to enhance growth and animals so supplemented actually lost weight and died sooner than did controls.

Fig. 1 depicts typical responses of rats to nicotinamide with

Rations K_{20} and K_{17}. Symptoms of general muscular atony and inanition were very apparent prior to death of all animals on Ration K_{20} plus the above compound. All animals on Ration K_{17} from the beginning, as well as the two which were shifted from Ration K_{20} to K_{17} at the time of introducing nicotinamide, grew uniformly and appeared entirely normal, although growth ranged to only about 10 gm. per week. Similar results were obtained with

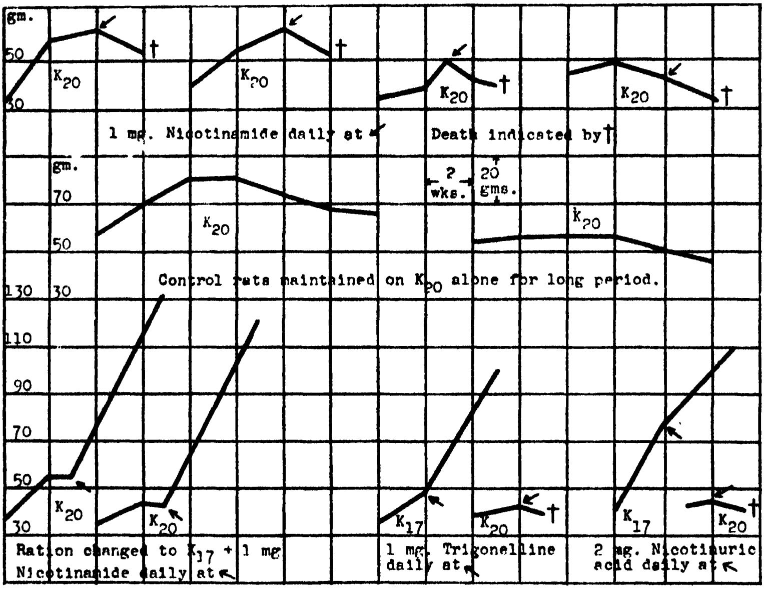


FIG. 1. Growth curves for young male rats on Ration K_{20} alone and with added nicotinamide, trigonelline chloride, and nicotinuric acid contrasted with growth curves of rats on Ration K_{17} plus the same compounds.

trigonelline and nicotinuric acid. It is interesting that these compounds give the same effect in rats, since trigonelline is inactive and nicotinuric acid is active in the treatment of black tongue in dogs.

Our results appear to indicate that inclusion of 12 per cent of white corn in Ration K_{20} supplies a factor, or factors, necessary to the proper utilization of nicotinic acid by the rat. Recently Fouts and Helmer (16) demonstrated the supplementary action of nicotinic acid for the growth of rats maintained on the Goldberger ration with added riboflavin. Without added riboflavin, however, nicotinic acid exercised an inhibitory effect on growth and raised the mortality above that of controls. That the addition of one, or two, or three members of the vitamin B complex may enhance deficiency symptoms of other members is not new knowledge in this field, but demands more attention in the future than it has received in the past.

Although differences in growth effects and in chemical properties appear to exist between Factor W and the pyridine compounds thus far studied, it is possible that one of them is a constituent of a more complex molecule. Because the interdependence of unknown factors for the growth of rats is not known and because the pyridine compounds appear to play some rôle in the nutrition of the rat, nicotinic acid has been added to the basal rations used in assay of Factor W in an amount calculated to supply about 1 mg. per day.

SUMMARY

1. Evidence of the weak acidic nature of Factor W has been obtained by partition between butanol and water at varying pH.
2. Factor W has been found to be soluble in phenol and aniline, but not in ether.
3. Failure to precipitate Factor W from alcohol or water with various precipitants has been described.
4. Successful elutions of Factor W from norit with various eluants have been made.
5. Evidence that Factor W is distinct from vitamin B_6, the chick antidermatitis factor, and nicotinic acid has been presented.
6. Results are presented to show that the response of rats to nicotinic acid and its amide is dependent upon the composition of the ration.

BIBLIOGRAPHY

1. Elvehjem, C. A., Koehn, C. J., Jr., and Oleson, J. J., *J. Biol. Chem.*, **115**, 707 (1936).
2. Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, **121**, 255 (1937).
3. Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, **115**, 557 (1936).
4. Edgar, C. E., and Macrae, T. F., *Biochem. J.*, **31**, 886 (1937).

5. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
6. Morgan, A. F., Cook, B. B., and Davison, H. G., *J. Nutrition*, **15**, 27 (1938).
7. Macrae, T. F., and Edgar, C. E., *Biochem. J.*, **31**, 2225 (1937).
8. Elvehjem, C. A., and Koehn, C. J., Jr., *J. Biol. Chem.*, **108**, 709 (1935).
9. Booher, L. E., *J. Biol. Chem.*, **119**, 223 (1937).
10. Schultz, H. W., and Mattill, H. A., *J. Biol. Chem.*, **122**, 183 (1937–38).
11. Oleson, J. J., Bird, H. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **127**, 23 (1939).
12. Woolley, D. W., Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Biol. Chem.*, **125**, 715 (1938).
13. Cook, C. A., Clarke, M. F., and Light, A. E., *Proc. Soc. Exp. Biol. and Med.*, **37**, 514 (1937).
14. Dann, W. J., *Science*, **86**, 616 (1937). Dann, W. J., and Subbarow, Y., *J. Nutrition*, **16**, 183 (1938).
15. Emerson, G. A., and Evans, H. M., *Proc. Soc. Exp. Biol. and Med.*, **38**, 195 (1938).
16. Fouts, P. J., and Helmer, O. M., *J. Nutrition*, **16**, 271 (1938).

EXPERIMENTS WITH THE FILTRATE FACTOR

By THOMAS H. JUKES

(*From the Division of Poultry Husbandry, College of Agriculture, University of California, Davis*)

(Received for publication, January 3, 1939)

The term "filtrate factor" was applied (1) to a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks. The specific dermatitis may be induced in chicks by feeding a diet of yellow corn-meal, wheat middlings, washed casein, minerals, and cod liver oil (2), or a heated diet of natural foodstuffs (3) or a simplified diet (1) low in the filtrate factor. Recently the terms "chick antidermatitis factor" (4) and "chick antidermatosis vitamin" (5) have also been used.

The term "Factor 2" has been applied to a fraction which has hitherto always been found present in filtrate factor concentrates and which is needed by the rat (6, 7). Factor 2 may or may not be identical with the filtrate factor. The filtrate factor has been shown to differ from vitamin B_6 (the rat antidermatitis factor, Factor 1) (6). Dann (8) demonstrated that the filtrate factor is not nicotinic acid; his findings have been confirmed (9). The present communication reports further experiments with the filtrate factor.

EXPERIMENTAL

The method of assay is the same as previously described (10), except that filtrate factor concentrates prepared from liver[1] or whey (11) often replaced rice bran filtrate in the positive control diet. The whey adsorbate always supplied at least 300 micrograms of riboflavin per 100 gm. of diet. The assay period varied from 6 to 14 days. Chicks with filtrate factor deficiency readily succumb to chilling, and they were hence allowed access to a temperature of 38° in their cages.

---

[1] Dr. H. W. Rhodehamel, Eli Lilly and Company, Indianapolis, kindly supplied the liver extract.

Alleviation of symptoms of dermatitis was always noted to be approximately proportional to growth when a potent supplement was added to the heated diet (Diet 80-G (10)). However, the method of assay is based on growth, and it was hence thought desirable to examine the diet for deficiencies in growth essentials *other than the filtrate factor. It was repeatedly noted that refined preparations of the filtrate factor, when fed at supraoptimal* levels, produced growth responses equal to those observed on the positive control diet. This was thought to indicate that the crude concentrates used in the positive control diet were probably effective only as sources of the filtrate factor, especially since in some cases the refined preparations (11) were found to be free from the factor promoting growth and preventing paralysis on a simplified diet (12) and also[2] from "Factor U" (13).

The possibility remains that Diet 80-G may be slightly deficient in growth essentials other than the filtrate factor, and that prolonged feeding periods would reveal such deficiencies. The use of depleted chicks and a short assay period should tend to minimize the effect of other deficiencies. Chicks that had been depleted on Diet 80-G for periods of 3 to 4 weeks responded within 2 days, as shown by a marked increase in weight, to administration of concentrates of the filtrate factor orally or by intraperitoneal injection. The use of such prolonged depletion periods, however, resulted in high mortality, and a depletion period of 7 to 9 days was customarily used.

It is known that a marked lowering of the nutritive value of casein results upon exposure to prolonged dry heat (14). Diet 80-G was therefore examined for protein deficiency as follows: 20 parts of unheated sardine meal were substituted for the casein and part of the corn-meal in the positive control diet. Sardine meal has been shown (15) to supply protein of biological value superior to that of casein for chicks. Ten chicks were used in each group. The gain on the positive control diet containing casein was 86 gm., on the diet containing sardine meal, 84 gm., and on the basal diet, 6 gm. At the end of the 15 day assay period no dermatitis was observed on the casein positive control or the sardine meal diets, while the syndrome score on the basal diet was 33. It was concluded that protein deficiency due to heat damage did not retard growth on the positive control diet during the assay period.

---

[2] Personal communication from Dr. E. L. R. Stokstad.

Table I summarizes a number of biological assays made in the customary way (10) on groups of from six to ten depleted chicks fed Diet 80-G.

TABLE I

Biological Assays on Depleted Chicks Fed Diet 80-G

Series No.	Length of assay period	Supplement to 100 gm. of basal diet	Gain during assay period	Syndrome score (16)
	days		*gm.*	
1	12	None	7	13
		0.25 cc. liver concentrate, heated in boiling water bath for 30 min. at pH 11	12	17
		0.25 cc. liver concentrate, heated as above at pH 10	14	10
		0.25 " " " " " " " " 9	27	2
		0.25 " " " untreated	47	5
		Positive control diet	63	0
2	14	None	10	14
		1 cc. arginine-free filtrate	63	0
		1 " " " + 0.8 gm. arginine	57	0
		Positive control diet	69	0
3	11	None	13	23
		Green barley shoots, equivalent to 40 gm. by fresh weight, dried on diet at room temperature	10	23
		Positive control diet	77	0
		" " " + 25% of hemp-seed meal	75	0

Series 1 indicated that virtually complete destruction of the filtrate factor was brought about by heating in aqueous solution at pH values of 10 and above. 50 per cent destruction took place at pH 9 under the conditions of the experiment. These results were reported previously in abstract form (17).

Series 2 indicated that no arginine deficiency was detectable in the basal diet, since growth was not improved by addition of arginine to an arginine-free filtrate. This finding is of some significance in view of the results of Arnold and coworkers (18), who found that growth of chicks on an unheated diet quite similar in composition to the basal heated diet in the present communication, supplemented with 1 per cent of yeast, was improved by the addition of arginine. Arginine-free filtrate was prepared as follows: Liver extract powder No. 343 (Lilly) was extracted with a solution of dry hydrogen chloride gas in isoamyl alcohol. The extract was concentrated under reduced pressure and taken up with water. The aqueous solution was partially decolorized by one treatment with adsorbent charcoal, followed by two treatments with filtrol (an adsorbent clay). The filtrate was treated with an excess of freshly precipitated lead hydroxide, the precipitate was rejected, and lead was removed from the filtrate by treatment with hydrogen sulfide. The filtrate was then treated with excess of silver sulfate followed by excess of barium hydroxide to remove arginine. The filtrate from this treatment was freed from silver by hydrogen sulfide and barium by sulfuric acid, and concentrated under reduced pressure. The preparation (arginine-free filtrate) reacted negatively to the Sakaguchi test for arginine. No apparent loss in activity resulted from the silver treatment, although Woolley and coworkers (4) have recently reported a loss of activity upon treatment of a concentrated preparation with silver oxide.

Series 3 indicated the absence of filtrate factor from green barley shoots, and showed that growth on the positive control diet was not improved during the assay period by the addition of hempseed meal.

A concentrate prepared from whey (11) and containing about 58 filtrate factor units (10) per cc. was subjected to the following treatment: The acidity of 50 cc. aliquots was adjusted to various pH values by addition of hydrochloric acid or sodium hydroxide. Appropriate indicators were used for the measurement of pH. The solutions were brought to 75 cc. by addition of water, and shaken with 150 cc. of acetone, which acted as an immiscible solvent. The acetone and aqueous phases were separated, neutralized, and concentrated to appropriate volumes. The result of assaying the solutions is shown in Fig. 1. Evidently the factor did not dissolve appreciably in acetone at pH values of 6 and above. This may indicate that the factor is an acid which is

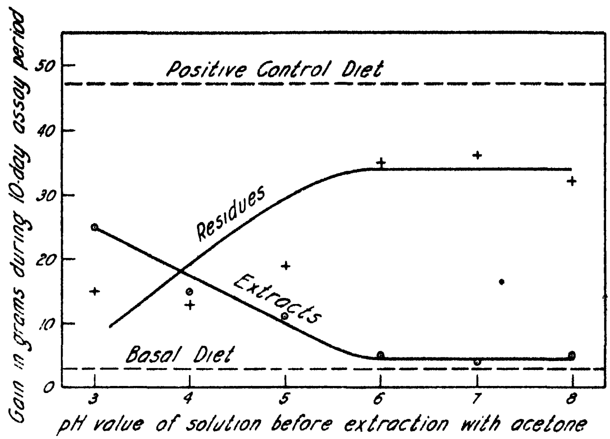


FIG. 1. Illustrating the results of extracting aqueous solutions of the filtrate factor with acetone. Each cross indicates the growth of a group of chicks fed an aqueous phase, and each circle indicates the growth of a group of chicks fed an acetone phase. All fractions were fed at a level corresponding to 1.2 per cent of the original solution. The upper dotted line indicates growth of a group of chicks on the positive control diet, and the lower dotted line indicates the growth of a group of chicks on the basal diet. From nine to eleven chicks were used in each of the fourteen groups of chicks in the experiment. The solid lines are arbitrarily sketched to illustrate the trends. The syndrome scores (16) for each group at the end of 13 days were as follows:

Supplement to basal heated diet	Syndrome score
3% of original solution (positive control diet)	0
Residue fraction from extraction with acetone at pH 8	2
“ “ “ “ “ “ “ “ 7	0
“ “ “ “ “ “ “ “ 6	0
“ “ “ “ “ “ “ “ 5	12
“ “ “ “ “ “ “ “ 4	17
“ “ “ “ “ “ “ “ 3	14
Extract “ “ “ “ “ “ “ 8	23
“ “ “ “ “ “ “ “ 7	21
“ “ “ “ “ “ “ “ 6	16
“ “ “ “ “ “ “ “ 5	13
“ “ “ “ “ “ “ “ 4	14
“ “ “ “ “ “ “ “ 3	5
None	28

almost completely dissociated at pH 6. If it is assumed that the dissociation is between 95 and 98 per cent at pH 6.0, then the dissociation constant is between 2×10^{-5} and 5×10^{-5}. The assumption seems reasonably justifiable from the data in Fig. 1. However, the addition of acetone may have caused some change in pH.

Destruction by Autoclaving—In an earlier report (19) it was stated that the factor was not destroyed in yeast by autoclaving. It has since been observed that the effect of autoclaving is variable, and that from 90 to 95 per cent of the potency of yeast is frequently destroyed by autoclaving at 120° for 5 hours. A sample of rice bran extract was rendered completely inactive by such a treatment.

Effect of Various Reagents—A concentrate was freed from chlorides by treatment with lead hydroxide followed by silver oxide. The solution was then acidified with sulfuric acid, and lead and silver were removed with hydrogen sulfide. The activity of an aliquot of the concentrate was completely destroyed by addition of a slight excess of potassium permanganate, but the addition of a corresponding amount of manganous sulfate to another aliquot did not destroy the activity.

Aliquots of a concentrate from which the bases had been removed by treatment with phosphotungstic acid were allowed to stand at room temperature for 2 hours in contact with (*a*) excess of sodium hydroxide, (*b*) excess of bromine and sodium hydroxide, and (*c*) excess of bromine in acid solution. The solutions were then neutralized and excess of bromine was removed by aeration. In no case could loss of activity be detected. Evidently heat is necessary to bring about inactivation in alkaline solution. Another preparation was treated with bromine in a slightly acid solution, and the excess of bromine was removed by aeration. Excess of barium hydroxide was then added, and the resulting precipitate was removed. The filtrate was concentrated under reduced pressure and poured into 8 volumes of 95 per cent ethanol. The resulting precipitate was removed. Both precipitates were found to be inactive, but the filtrate retained most of the original activity of the concentrate.

Copper acetate, followed by excess of sodium hydroxide, was added to an active preparation. The precipitate and filtrate were separated and copper was removed by treatment with hydrogen sulfide. The precipitate fraction was inactive, and the filtrate fraction retained the potency of the original preparation.

Attempts were made to precipitate the factor with mercuric ion as follows: Ethanol extracts of rice bran, liver, yeast, and whey concentrates were prepared and a solution of mercuric chloride in ethanol was added, followed by excess of potassium hydroxide dissolved in ethanol. The precipitate and filtrate fractions were acidified and freed from mercury by treatment with hydrogen sulfide. With one exception, the entire original activity was found in the filtrate fraction. The exception was a concentrate prepared from whey. In this case the activity was equally divided between the filtrate and precipitate, and a repetition gave similar results. Other preparations from whey retained the entire activity in the filtrate fraction. Treatment with cadmium chloride in ethanol solution did not precipitate the factor from an ethanol extract of a whey concentrate. Addition of excess of potassium hydroxide dissolved in ethanol to the filtrate from this treatment did not precipitate the factor, which remained in the filtrate.

DISCUSSION

Marked progress in concentration and purification of the chick antidermatitis (filtrate) factor has been achieved by Woolley and coworkers (4). These investigators concluded that the factor is acidic. The distribution of the factor between amyl alcohol and water has been previously shown to be influenced by the acidity of the aqueous phase (20, 1), which finding leads to a similar conclusion with regard to the acid nature of the factor. In the present investigation the distribution of the factor between acetone and water was studied, and the results indicated that the factor may be a comparatively strong organic acid. To judge from its versatile solubility, it is of low molecular weight. Its marked resistance to bromination and its failure to form insoluble metallic compounds in aqueous or alcoholic solutions are interesting properties.

The factor is present in grains (10, 19), but could not be detected in some cases in certain young green leaves such as barley shoots and lawn clippings (21). In this respect it may be contrasted with riboflavin, which is present in low concentration in grains, but in fairly high concentration in young green leaves. However, the filtrate factor content of green leaves is variable.

SUMMARY

1. The filtrate factor (chick antidermatitis factor) was found to be destroyed in alkaline solution at 100°, but was not destroyed at room temperature. It was destroyed by potassium permanganate in acid solution. Autoclaving yeast or rice bran extract at 120° for 5 hours had a destructive action on the factor.

2. Unsuccessful attempts were made to form insoluble compounds of the factor with the following metallic ions, silver, mercuric, cupric, and cadmium.

3. The factor was not destroyed by the action of bromine in acid or in alkaline solution.

4. Aqueous solutions of varying degrees of acidity were extracted with acetone. The factor remained in the aqueous phase at acidities corresponding to pH 6.0 and above, but passed into the acetone phase when the aqueous solution was more acid than pH 6.0. This is thought to indicate that the factor is an acid which is dissociated almost completely at pH values of 6.0 and above, and hence is a comparatively strong organic acid.

The advice of Dr. S. Lepkovsky is gratefully acknowledged.

BIBLIOGRAPHY

1. Lepkovsky, S., and Jukes, T. H., *J. Biol. Chem.*, **114**, 109 (1936).
2. Ringrose, A. T., Norris, L. C., and Heuser, G. F., *Poultry Sc.*, **10**, 166 (1931).
3. Kline, O. L., Keenan, J. A., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **99**, 295 (1932-33).
4. Woolley, D. W., Waisman, H. A., Mickelsen, O., and Elvehjem, C. A., *J. Biol. Chem.*, **125**, 715 (1938).
5. Bauernfeind, J. C., Schumacher, A. E., Hodson, A. Z., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, **39**, 108 (1938).
6. Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, **115**, 557 (1936).
7. Lepkovsky, S., *J. Biol. Chem.*, **124**, 125 (1938).
8. Dann, W. J., *Science*, **86**, 616 (1937).
9. Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **37**, 405 (1937).
10. Jukes, T. H., *J. Biol. Chem.*, **117**, 11 (1937).

11. Lepkovsky, S., Taylor, L. W., Jukes, T. H., and Almquist, H. J., *Hilgardia*, **11**, 559 (1938).
12. Jukes, T. H., and Babcock, S. H., Jr., *J. Biol. Chem.*, **125**, 169 (1938).
13. Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, **125**, 687 (1938).
14. Greaves, E. O., Morgan, A. F., and Loveen, M. K., *J. Nutrition*, **16**, 115 (1938).
15. Almquist, H. J., Stokstad, E. L. R., and Halbrook, E. R., *J. Nutrition*, **10**, 193 (1935).
16. Lepkovsky, S., and Jukes, T. H., *J. Biol. Chem.*, **111**, 119 (1935).
17. Lepkovsky, S., and Jukes, T. H., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **119**, p. lx (1937).
18. Arnold, A., Kline, O. L., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **116**, 699 (1936).
19. Jukes, T. H., and Lepkovsky, S., *J. Biol. Chem.*, **114**, 117 (1936).
20. Elvehjem, C. A., and Koehn, C. J., Jr., *J. Biol. Chem.*, **108**, 709 (1935).
21. Jukes, T. H., *J. Nutrition*, **14**, 223 (1937).

THE B VITAMINS AND FAT METABOLISM

II. THE EFFECT OF THIAMINE UPON THE SYNTHESIS OF BODY FAT IN PIGEONS

By E. W. McHENRY and GERTRUDE GAVIN

(*From the School of Hygiene, University of Toronto, Toronto, Canada*)

(Received for publication, January 3, 1939)

The hypothesis has been proposed (1) that one of the functions of thiamine is the synthesis of fat from carbohydrate. In a previous paper (2) we have reported the apparent production of fat from carbohydrate in rats which had been fed thiamine, and that the amount of fat was further increased by the addition to the diets of two other B vitamins. For many years pigeons have been classical test subjects for thiamine investigations. Accordingly, it seemed advisable to investigate the effect of thiamine on the formation of fat from carbohydrate in this species.

Methods

Normal adult pigeons, bought in the open market, were used. They were kept in individual screen bottom cages with food and water available at all times. The pigeons were weighed twice weekly. The diet consisted solely of polished rice. Merck's crystalline thiamine dissolved in a 4 per cent solution of ethyl alcohol was administered *per os* as the only supplement. The criteria of the presence of polyneuritis were loss of ability to walk and fly and the development of head retraction. The pigeons were killed by ether inhalation, plucked, and the bodies analyzed for total crude fatty acids according to the method previously published (2). The results are expressed as the percentage of total crude fatty acids in the body after removal of the feathers.

EXPERIMENTAL

Series I—In order to obtain the normal value of the total crude fatty acids in the bodies of pigeons three groups of birds were investigated. One group was killed at the start of the experiment, the second was fed whole corn for 1 week, and the third was fed polished rice for 1 week. No supplements were given. The average weights of the pigeons and the content of crude fatty acids in the bodies are given in Table I.

Series II—The body fat in pigeons suffering from polyneuritis was compared with that of a group receiving thiamine. One group of pigeons was fed polished rice, *ad libitum*, until polyneuritis developed, and during the same period the other group received 60 micrograms of thiamine daily in addition to the rice *ad libitum*. This supplement completely protected the pigeons against polyneuritis. The average crude fatty acid value of the bodies is given in Table I. The body fat had fallen to a very low level in the polyneuritic group. In contrast, the thiamine-fed birds showed a value almost double that of the normal birds, determined in Series I.

TABLE I

Effect of Thiamine Supplements upon Body Weight and Body Fat

	No. of pigeons	Average weight	Total crude fatty acids	
		gm.	*per cent*	*gm.*
Series I				
At start of experiment	4	267	7.9	21.1
Corn diet 1 wk.	4	275	8.1	22.3
Polished rice diet 1 wk.	4	269	9.7	26.0
Series II				
Polyneuritic	10	168	3.2	5.4
Control, fed thiamine	10	286	16.5	47.2
Series III				
At start of experiment	10	315	6.0	18.9
After 18 days depletion	10	247	8.4	20.7
Polyneuritic	10	182	2.1	3.8
Thiamine supplied after 18 days depletion	10	303	17.4	52.7

Series III—The high body fat of the thiamine-fed birds in Series II might be considered as merely conservation of fat already present in the body. Series III was designed to show that thiamine would cause the synthesis of fat to occur, even if the thiamine was given after a preliminary depletion period. For this experiment four groups of pigeons of the same average weight were investigated. The first group was killed at the start of the experiment to serve as a normal control. The other three groups were fed polished rice throughout the experiment. A second group was killed after 18 days, at which time the body stores of thiamine are probably depleted. Of the remaining two groups, one was continued on the rice diet until polyneuritis developed and the other was given, in addition to the rice, 60 micrograms of thiamine daily for the same number of days. The average weight and crude fatty acid content of the bodies are given in Table I. The weight of fatty acids in the bodies is calculated for the bodies less feathers.

Table II

Effect of Thiamine on Pigeons

All figures are averages for ten birds.

	Polyneuritic pigeons	Controls (+ thiamine)
	gm.	*gm.*
Initial weight	460	460
Final weight	264	431
Polished rice intake per pigeon	386	835
Fat in ingested rice	1.1	2.5
Crude fatty acids in body	9.5	67.8

Series IV—In view of the marked difference in body fat between pigeons suffering from polyneuritis and the birds receiving a supplement of thiamine, the polished rice eaten by two such groups was measured in order to estimate the amount of fat supplied directly by the rice. In this series the average time for the development of polyneuritis was 42 days. A pigeon receiving thiamine from the start of the experiment was killed at the same time as the polyneuritic one. The amount of food eaten, the initial and final weights, and the amount of crude fatty acids in the bodies at the end of the experiment are given in Table II.

DISCUSSION

During the development of thiamine deficiency and subsequent polyneuritis pigeons lose appreciable weight. In Series III during the preliminary 18 day depletion period there was a considerable fall in body weight, and a further reduction occurred in those birds which developed polyneuritis. In contrast, the pigeons which received thiamine after the preliminary depletion period had almost regained their initial weight. In Series IV in which thiamine was given to one group of pigeons during the entire experiment, the loss in weight of these was slight compared to that of the deficient birds.

During the development of polyneuritis the content of crude fatty acids in the body fell from an initial value of 8 to 10 per cent to 2 to 4 per cent. If the change in body weight was considered (Series III), the total body fat fell from 18.9 gm. to 3.8 gm. In this series there was no significant change in body fat during the preliminary depletion period, although there was a marked loss in weight. From then until polyneuritis developed, a period of 2 to 4 weeks, there was a marked decrease in body fat, indicating that the stores of body fat are not depleted until there has been a considerable lowering of the content of thiamine in the body. However, if after the 18 day depletion period thiamine was given, the body fat rose rapidly, showing a final value of 52.7 gm. Of the average gain in weight of 56 gm. during the supplemental period, 32 gm. or 56 per cent was accounted for by the increase in crude fatty acids in the body. Since thiamine was the only supplement given, it must have caused this increase in body fat.

The only possible source of new fat was the polished rice eaten. From Series IV the amount of fat, as such, present in the rice consumed can be calculated. In this experiment the pigeons were exceptionally large at the beginning of the experiment and required the longest time of any series to develop polyneuritis. In spite of this, the difference in food intake between the pigeons fed the thiamine supplement and the polyneuritic pigeons was 449 gm. of rice, which would supply 1.4 gm. of fat as such. The difference in the final crude fatty acid content for these two groups was 58.3 gm. Thus it is clear that the amount of fat supplied in the rice was insignificant. The remainder of the fat must have been synthesized from the polished rice, most probably from the carbohydrate contained in it.

The suggestion might be advanced that experiments of this type do not demonstrate that thiamine causes a synthesis of fat but that such synthesis takes place under conditions of adequate nutrition. On this basis fat synthesis would be limited by a deficiency of any essential dietary constituent and would be restored to a normal amount by supplying the deficient factor. Unpublished data from this laboratory (Sheppard and McHenry) show that guinea pigs, deficient in ascorbic acid, have a greater body fat than paired fed animals receiving ascorbic acid. In this case, at least, a dietary deficiency of another type has not caused a condition similar to that seen in thiamine deficiency.

We regard the reported experiments as further evidence in favor of the correctness of the hypothesis of thiamine function; namely, the synthesis of fat from carbohydrate.

SUMMARY

It has been found that thiamine, as the only supplement to a polished rice diet, causes an increase in content of crude fatty acids in pigeons, presumably by synthesis from carbohydrate.

BIBLIOGRAPHY

1. McHenry, E. W., *Science*, **86**, 200 (1937).
2. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).

XANTHINE OXIDASE: PURIFICATION AND PROPERTIES*

BY ERIC G. BALL†

(From the Kaiser Wilhelm-Institut für Zellphysiologie, Berlin-Dahlem, Germany)

(Received for publication, December 29, 1938)

The oxidation of hypoxanthine and xanthine to uric acid in the presence of tissue brei and oxygen was first recognized in 1899 by Spitzer (11) as an enzymatic process. The enzyme involved was named xanthine oxidase by Burian (3). In 1902 Schardinger (10) described a reaction in which the reduction of methylene blue by aldehyde was catalyzed by the presence of fresh milk, the reaction and enzyme involved being named after the discoverer. These two enzymatic processes remained unrelated until some 20 years later when Morgan, Stewart, and Hopkins (9) showed that milk was a rich source of xanthine oxidase. This finding immediately raised the question of the identity of xanthine oxidase and the Schardinger enzyme. From a study of the various factors affecting these two reactions most workers (*cf.* Dixon and Thurlow (7), Booth (2)) have reached the conclusion that the same enzyme is involved in both. It is the purpose of this paper to describe the isolation of an enzyme preparation from whole milk which catalyzes both of these reactions and which is 500 times more active per unit of dry weight than the starting material. Evidence will be presented which indicates that this enzyme is a flavoprotein. The properties of this preparation and those of its flavin prosthetic group will be described.

---

* A preliminary report of this work was made before the Twenty-second International Physiological Congress at Zurich, August 14–19, 1938, and in *Science* (1).

† Fellow of the John Simon Guggenheim Memorial Foundation on leave of absence from the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore.

Enzyme Preparation

The activity per unit of dry weight of the enzyme preparation during the process of purification was followed by determining manometrically at 20° the oxygen consumption with hypoxanthine as the substrate. The manometric test was arranged as follows: The main chamber of a conical manometric flask contained 1.0 ml. of 0.1 M phosphate buffer of pH 7.2, 0.1 to 0.5 ml. of the enzyme solution, and enough water to make a total volume of 3.0 ml. The center well contained 0.2 ml. of 5 per cent KOH merely as a precaution, since no CO_2 is developed during the oxidation. The side bulb contained 0.2 ml. of a 0.05 M solution of hypoxanthine in 0.05 M NaOH. The pH of the final mixture after the alkaline substrate was tipped in was 7.5 as determined by the glass electrode. The gas chamber contained air, the rate of oxygen consumption with pure oxygen being only 10 per cent higher. Dry weights were determined by calculation from protein nitrogen determination or by direct estimation on a dialyzed preparation. Activity is expressed below in terms of Q_{O_2}, *i.e.*, the c.mm. of O_2 absorbed per hour per mg. of dry weight, readings during the first 30 minutes of the reaction being employed.

Preliminary experiments with fresh whole milk showed that a large part of its xanthine oxidase content was associated with the cream fraction. The portion in the skim milk fraction increased as the milk aged, especially at low temperatures. This observation furnished the working hypothesis that the enzyme was adsorbed on the fat droplets and could be forced into solution if the adsorption surface was decreased by causing the fat droplets to coalesce. This hypothesis, the correctness of which is not vouched for, forms the basis of the following method. The procedure possesses the advantage that the large bulk of the milk proteins is disposed of immediately. It is obvious that fresh whole milk or cream should be employed.

Procedure

Step I—Fresh whole milk ($Q_{O_2} = 0.5$) is centrifuged and the cream layer sucked off and recentrifuged. As complete a removal of the milk proteins as is possible is desirable. To each 100 gm. of cream, 100 ml. of 0.2 M Na_2HPO_4 are added and the mixture is warmed to 38° and shaken gently at this temperature on a shaking machine for 2 hours. The mixture is then centrifuged, preferably at 0° so that the fat solidifies, and the aqueous portion is drawn off from under the fat. The fat is discarded, as a second extraction is not worth while. The last traces of fat are removed from the enzyme-containing extract by recentrifuging. The extract so obtained (120 to 130 ml.) has a milky appearance, and contains considerable insoluble organic matter which has the appearance of cellular débris. Removal of this insoluble material by high speed centrifugation diminishes its xanthine oxidase activity. The pH of the extract is 7.5, its Q_{O_2} 7 to 10. Yield, 50 per cent of whole milk. Though other salt solutions may be used to extract the enzyme from cream, the requirements of Step II dictate the use of the alkaline disodium phosphate solution.

Step II—To each 100 ml. of the above extract, 100 mg. of a commercial preparation of pancreatic lipase are added and the mixture incubated at 38° for 3½ hours. No preservative is used. The mixture which is still turbid is cooled to 20°. Addition of 0.5 M $CaCl_2$ to this solution, which is rich in phosphate, produces a copious precipitate of calcium phosphate which after being centrifuged off leaves a clear yellowish solution of the enzyme. No more $CaCl_2$ should be added than is necessary to produce a clear supernatant or the yield will suffer. The $CaCl_2$ should be added slowly with thorough shaking and the mixture allowed to stand 15 minutes before centrifuging. Usually 16 to 20 ml. of $CaCl_2$ (0.5 M) to 100 ml. of digest are required. The amount needed, however, is best determined for each batch by a pilot run; *e.g.*, 2.5 ml. of digest are treated with 0.2, 0.3, 0.4, and 0.5 ml. of 0.5 M $CaCl_2$. The pH of the supernatant is 6.2, its Q_{O_2} 25. Yield, 75 per cent.

The mode of action of lipase in this step is not clear. Its employment (or crude commercial trypsin) in conjunction with adsorption on freshly precipitated calcium phosphate has been the only method found of removing troublesome turbidities at this stage. If a batch still possesses some turbidity after this treatment, it will be found that this is removed during the subsequent steps.

Step III—The above solution is 60 per cent saturated with ammonium sulfate and allowed to stand at 0° overnight. The precipitate is centrifuged off at 0° and dissolved in a measured volume of water equal to one-tenth that of the original solution.

The volume of the resulting solution is measured and the volume of the precipitate obtained from the difference. (See Step IV.) This solution is golden brown in color, sometimes slightly turbid, its pH is 5.7, $Q_{O_2} = 100$. Yield, 90 per cent.

Step IV—Ammonium sulfate is added to the above solution to bring its total concentration to 33 per cent saturation. The amount of $(NH_4)_2SO_4$ already present in solution is calculated, assuming that the volume of precipitate in the above step is all 60 per cent ammonium sulfate. The precipitate is centrifuged off at 0° and discarded and the supernatant brought to 42 per cent saturation with ammonium sulfate. The resulting precipitate is centrifuged down at 0° and dissolved in an amount of water equal to the volume of the original solution. The solution so obtained is a clear golden brown color, its pH is 5.4, $Q_{O_2} = 200$. Yield, 75 per cent. Such a preparation has been used for the majority of experiments described below.

By further ammonium sulfate fractionation it is usually possible to effect a further slight increase in purity. The purest preparation so obtained possessed a Q_{O_2} value of 270. Though this preparation appeared to be fairly pure, various methods of precipitation at its isoelectric point yielded at best only a semicrystalline product.

The above procedure may be interrupted at the end of each step for at least 1 week without loss in activity of the product, provided it is kept at 0°. The final product is best preserved in the precipitated state under ammonium sulfate at 0°. So kept, it shows little loss of activity over several months.

1 liter of whole milk yields about 40 mg. of final enzyme preparation. Since the over-all yield of the process is about 25 per cent, whole milk contains about 160 mg. per liter of xanthine oxidase of this purity.

Absorption Spectra of Enzyme

Solutions of the enzyme have a strong golden brown color. In Fig. 1 the curve labeled "oxidized" is the absorption spectrum of a solution buffered at pH 7.4 with 0.1 M phosphate and containing 6.25 mg. per ml. of an enzyme preparation of Q_{O_2} 191. The apparatus and technique employed were similar to those described in earlier publications from this laboratory (*cf.* Warburg (15)).

In the visible region there exists one broad band lying between λ 400 to 500 mμ. In the ultraviolet there is present a sharp band centered at λ 275 mμ, characteristic of most protein solutions. The ratio of the height of these two bands is approximately 12, which is almost identical with that for the two similarly situated bands of the pure yellow enzyme (Haas, unpublished data). The height of the absorption band at λ 455 mμ permits a calculation of the maximum molecular weight of xanthine oxidase if we assume that its molar absorption coefficient at this wave-length is identical with that of the yellow enzyme at λ 450 mμ; namely, 2.4 × 10^7 sq. cm. per mole. Taking the nitrogen content of xanthine oxidase as 16 per cent, we obtain a value of 100,000.

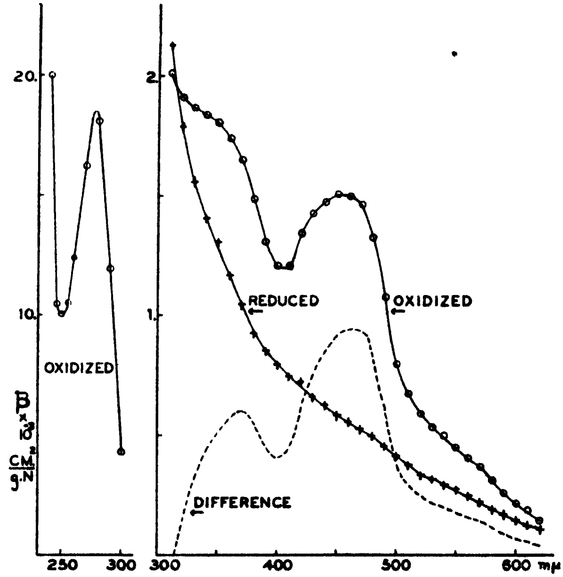


Fig. 1. Absorption spectra of the enzyme preparation. The solution measured contained 6.25 mg. per ml. of an enzyme preparation of Q_{O_2} = 191, pH 7.4. Reduction was performed by the addition of 0.2 ml. of 0.05 M hypoxanthine to 5.8 ml. of the enzyme solution in the absence of air.

If the substrate hypoxanthine is now added to this enzyme solution in the absence of air, the color fades but does not entirely disappear even if an excess of hypoxanthine is added. The absorption spectrum of the resulting solution is the curve in Fig. 1 labeled "reduced." A further slight diminution in color may now be produced by the addition of dithionite ($Na_2S_2O_4$). If air is then admitted, the original color is fully restored. The process can be repeated.

If the values for the absorption curve of the reduced form of the enzyme are subtracted from those of the oxidized form, we obtain the curve in Fig. 1 labeled "difference." This curve shows two absorption bands centered at λ 465 mμ and λ 370 mμ respectively and resembles more nearly those of other flavoproteins whose oxidized forms show two distinct bands in these regions.

Preparation and Properties of Flavin Prosthetic Group

The prosthetic group can be completely split off from the protein by acidification, heat, or the addition of alcohol. The protein is denatured in the process and forms an insoluble white precipitate. The solution that remains after removal of the protein precipitate is a pure yellow color which is in marked contrast to the golden brown color of the enzyme solutions. This solution fluoresces strongly in ultraviolet light. Its color can be completely discharged by the addition of dithionite and subsequently restored by shaking with air. The yellow pigment which is chloroform-insoluble can be converted into a chloroform-soluble lumiflavin derivative in 75 per cent yield by irradiation in alkaline solution according to the method of Warburg and Christian (16). The absorption spectrum of the product so obtained leaves no doubt as to the flavin nature of the pigment, as it is identical with that given by Warburg and Christian for the lumiflavin obtained by them from the yellow enzyme.

The absorption spectrum of the prosthetic group itself, which is shown in Fig. 2, is clearly that of a flavin. This particular sample was prepared as follows: To 1 volume of enzyme solution there were added 4 volumes of methyl alcohol. The protein precipitate was centrifuged off and washed with methyl alcohol and then discarded. The yellow methyl alcohol solution of the flavin was then evaporated to dryness under a vacuum at 40° and the yellow residue redissolved in the smallest possible amount of absolute CH_3OH, an insoluble white residue being centrifuged off and discarded. This alcoholic solution was evaporated to dryness as before and the residue dissolved in water. This solution was then made acid to Congo red with HNO_3 and the flavin precipitated as the silver salt by the addition of 30 per cent $AgNO_3$. The precipitate which was centrifuged off was washed twice at the centrifuge with a small amount of water. It was then suspended in water and the precipitate decomposed by H_2S. The precipitate of AgS was removed and the solution freed from H_2S. 5 ml. of this solution when analyzed by Herr Luttgens showed a content of 30.5 micrograms of phosphorus. This corresponds to a concentration of 1.97×10^{-7} moles of P per ml. The flavin concentration of the same solution was determined spectrophotometrically, assuming that the molar absorption coefficient β for λ 450 mμ was the same as that for riboflavin; namely, 2.6×10^7 sq. cm. per mole. A flavin concentration of 0.92×10^{-7} moles per ml. was found. Thus the concentration of phosphate is twice that of flavin, which suggests the presence of a dinucleotide structure perhaps similar to the flavin adenine dinucleotide reported by Warburg and Christian (17) as the coenzyme of the amino acid oxidase. In fact it is possible that this preparation is

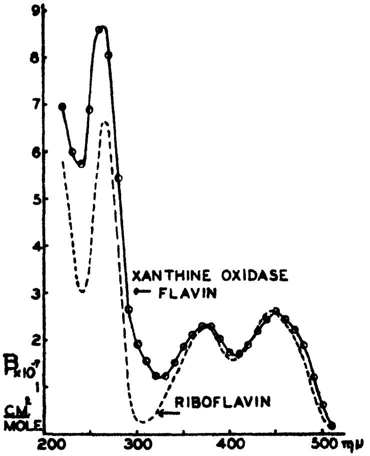


FIG. 2. Absorption spectra of the prosthetic group. The data have been plotted in terms of the molar absorption coefficient by making the assumption that the β value for xanthine oxidase flavin at λ 450 mμ is identical with that for riboflavin at λ 445 mμ. See text for preparation.

identical with the amino acid oxidase coenzyme. First their absorption spectra show a marked similarity. They are practically identical in the visible region, where both possess a maximum at λ 450 mμ. This is in contrast to riboflavin with its maximum at λ 445 mμ (see Fig. 2). Both also show an equal absorption at λ 265 mμ, an absorption which exceeds that of riboflavin in this region by an amount equal to that attributable to the presence of 1 adenine molecule in the compounds. Only in the band centered at λ 375 mμ do they differ. Here the spectrum of the coenzyme of the amino acid oxidase is lower even than that for riboflavin. Second, Professor Warburg informs me that this preparation is as active per unit of flavin concentration as the amino acid oxidase coenzyme in the test utilized by Warburg and Christian (17) in its isolation.

It might therefore appear that the active group of xanthine oxidase is a flavin adenine dinucleotide identical with the amino acid oxidase coenzyme. This, however, does not seem to be the case, as the following experiment will indicate. A solution of xanthine oxidase was placed in a cellophane sac and dialyzed against running distilled water at 0° for 2 weeks. At the end of this time the solution had lost most of its color and showed only about 20 per cent of its original activity. A solution containing the flavin was now prepared by simply heating an enzyme solution to 80° for 10 minutes and filtering off the denatured protein. This solution which alone was inactive, when added to the dialyzed preparation restored fully its original activity. In Fig. 3 are portrayed the results of an experiment showing the effect on the oxygen consumption of a dialyzed preparation upon addition of varying amounts of this flavin solution. The flavin concentration of the added solution was determined spectrophotometrically. Upon addition of the flavin solution the activity increased rapidly and reached a plateau value in this experiment after the addition of approximately 0.5×10^{-8} moles. A series of experiments was now run in which the same amount of dialyzed preparation as above was employed but 0.5×10^{-8} moles of the following flavins were substituted: riboflavin phosphate, amino acid oxidase coenzyme, and the flavin dinucleotide component of the yeast yellow enzyme isolated by Haas (8). These were all devoid of action. Also inactive was the xanthine oxidase flavin preparation described above that was prepared by alcohol precipitation and purified through the silver salt.

It therefore seems reasonable to conclude that the active coenzyme group of xanthine oxidase is not identical with any flavin compound hitherto known. The results might further be interpreted to indicate that a flavin plays no part in xanthine oxidase action. This may indeed be the case, though in the face of the other evidence it seems more likely that we are dealing with an active group composed of two components, or even with two different compounds, one of which is identical with the flavin adenine dinucleotide of Warburg and Christian and the other unknown but which is lost or destroyed when the flavin part is isolated by means of alcohol and silver salt precipitation. One wonders if perhaps a tri- or tetranucleotide type of compound is involved. An answer may be furnished by further work that is in progress.

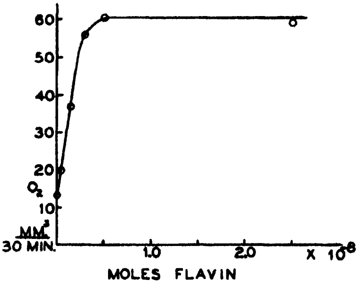


FIG. 3. Reversible splitting of protein and active group. The O_2 consumption of a dialyzed xanthine oxidase preparation (0.57 mg.) as affected by the addition of the flavin prosthetic group. Substrate hypoxanthine, pH 7.5, temperature 20°. The manometric procedure as described under "Enzyme preparation" was used.

From the experimental results shown in Fig. 3 it is possible to make a rough calculation of the maximum molecular weight of the enzyme. The quantity of dialyzed enzyme used here had a dry weight of 0.57 mg. and its nitrogen content as determined by Herr Luttgens was 16.3 per cent. In order to produce a maximum oxygen uptake with this quantity of protein it was necessary to add approximately 0.5 × 10^{-8} moles of flavin. We may therefore conclude that this amount of flavin is just sufficient to saturate all the protein present. However, since the protein contains a residue of flavin equal to about 20 per cent of its saturated value, the total amount of flavin in combination with 0.57 mg. of protein is 0.6×10^{-8} moles. If we now assume that each molecule of protein combines with 1 molecule of the flavin compound, then 0.57 mg. of protein must correspond to 0.6×10^{-8} moles. Hence a maximum molecular weight of 95,000 can be calculated. This preparation is undoubtedly impure, however, as it possesses a Q_{O_2} of 210. As stated earlier, the purest preparation obtained had a Q_{O_2} of 270, which would mean that such a preparation on this basis would yield a molecular weight of $210/270 \times 95{,}000$, or 74,000. The yellow enzyme according to Theorell (14) possesses a molecular weight of about 73,000.

Isoelectric Point

The isoelectric point of xanthine oxidase was determined by the method of Theorell (13). The cataphoresis experiments were performed at 0°. The determination of pH was made, however, at 20° with the glass electrode. No attempt has been made to correct the pH values to 0°, as the accuracy of the cataphoresis method did not seem to warrant it. The results which are plotted in Fig. 4 show that the isoelectric point lies at about pH 6.2. It is of interest to note that at each pH the migration of xanthine oxidase as determined manometrically with either hypoxanthine or aldehyde as substrate and the observed movement of colored material were always of the same direction and magnitude, an observation that lends support to the belief that xanthine oxidase and the Schardinger enzyme are one, and that that enzyme is a flavoprotein.

Oxidation of Aldehyde

There is a marked difference in the course of the oxidation of acetaldehyde as compared to hypoxanthine in the presence of the enzyme. Typical results obtained with these two substrates are shown in Fig. 5. Here the manometric procedure was the same as described for the isolation of the enzyme except that no KOH was employed in the center well. Although the initial rate of oxidation for both substrates in this experiment is almost the same, it rapidly falls off in the case of aldehyde and the reaction soon ceases. This is apparently due to the destruction of the enzyme, since the addition of more enzyme causes a further oxygen uptake. The addition of further substrate, aldehyde or hypoxanthine, is without effect.

The improbability of the oxidation of two substrates so chemically dissimilar as aldehyde and hypoxanthine by one and the same enzyme has long been voiced as an objection to the claim that xanthine oxidase and the Schardinger enzyme are identical.

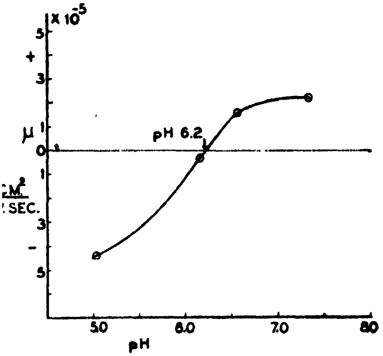


Fig. 4

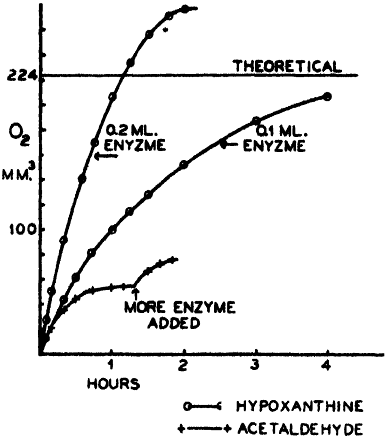


Fig. 5

Fig. 4. Isoelectric point of xanthine oxidase as determined by cataphoresis.

Fig. 5. Oxidation course for hypoxanthine and acetaldehyde. Temperature 20°, pH 7.5, substrates: 0.2 ml. of 0.05 M hypoxanthine, enzyme as indicated; 0.2 ml. of 0.10 M acetaldehyde, 0.1 ml. enzyme added at start and at point indicated; total volume 3.2 ml.

It seemed possible, however, that this apparent anomaly might be explained by assuming that before aldehydes underwent oxidation they first combined with amino groups present to form a linkage of the type $—\overset{H}{C}{=}N—$, a grouping identical with that which is attacked in hypoxanthine. To test this hypothesis, therefore, glycine was added to the reaction mixture in the expectation that an acceleration of the process or a protection of the enzyme from destruction might occur. The results were negative. The possibility still remains, however, that the destruction of the enzyme during oxidation of aldehydes may be due to a reaction of this type.

As can be seen from the curves in Fig. 4 the course of the oxidation of hypoxanthine is also not linear with time. The reaction, however, does run to completion and its rate is proportional to the enzyme concentration. As pointed out by earlier workers the total oxygen consumption is greater than the theoretical, owing to the formation of H_2O_2.

Action of Cyanide

The addition of cyanide alone to xanthine oxidase causes its irreversible inhibition. If, however, cyanide and substrate are added simultaneously, no inhibition occurs. This strange effect was first noted by Szent-Györgyi (12) and was later more extensively studied by Dixon and Keilin (6). The xanthine oxidase preparation described here behaves in a similar manner when treated with cyanide. A typical experiment is shown in Fig. 6. The extent of inhibition varies not only with the final concentration of cyanide as shown here but also with the length of time of contact of the cyanide and enzyme before the addition of substrate. The course of the oxidation in the presence of cyanide appears to be more linear with respect to time than in the control. This is perhaps best seen by comparison of Curve 1, where cyanide and substrate were added simultaneously, with Curve 2, the control.

The mode of action of cyanide remains as yet unexplained. It apparently, however, attacks the protein part of the enzyme and not the flavin coenzyme. Incubation of cyanide with the active flavin coenzyme does not impair in the slightest its ability to function when added to a dialyzed enzyme preparation as in the type of experiment shown in Fig. 3. The dialyzed enzyme preparation, however, on treatment with cyanide no longer responds to the addition of the flavin coenzyme preparation.

End-Product of Hypoxanthine Oxidation

According to previous workers the oxidation in air of hypoxanthine or xanthine in the presence of xanthine oxidase has yielded uric acid. Morgan, Stewart, and Hopkins (9) were able to isolate uric acid in 44 per cent of the theoretical yield using hypoxanthine as the substrate and whole milk as a source of the enzyme. An attempt to confirm these findings by allowing uricase to act upon the end-product of hypoxanthine oxidation gave unexpected results. In Fig. 7 the curve drawn with a solid line shows a representative experiment. Hypoxanthine was shaken in a manometer flask with xanthine oxidase until no further oxygen uptake was observed, the end-value being somewhat greater than the theoretical calculated for the production of uric acid. There was then added a sample of purified uricase as prepared by

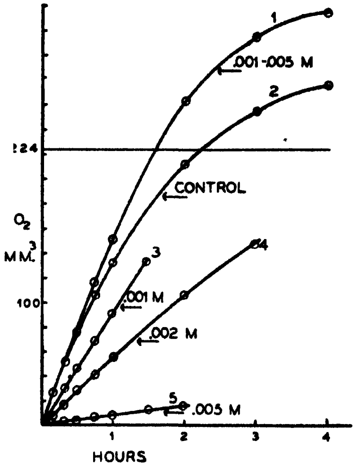


Fig. 6

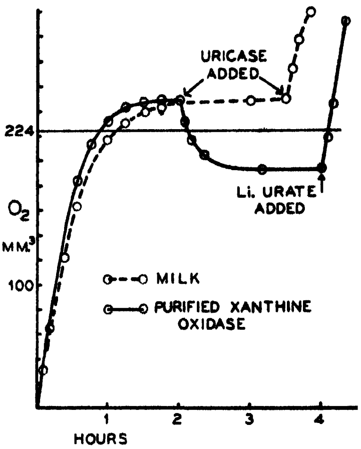


Fig. 7

Fig. 6. Action of KCN. Concentrations given are for the end-concentration of KCN in the flask. Temperature 20°, pH 7.5, substrate 0.2 ml. of 0.05 M hypoxanthine, theoretical oxygen consumption 224 c.mm., total volume 3.2 ml. The manometric procedure as described under "Enzyme preparation" was used except that no KOH was employed. Curve 1, KCN added simultaneously with substrate; Curve 2, control, no KCN; Curves 3 to 5, KCN in contact with the enzyme 1 hour at 20° before the substrate was added.

Fig. 7. Action of uricase on the end-product of hypoxanthine oxidation by crude and purified xanthine oxidase. Temperature 38°, pH 7.5, theoretical oxygen consumption for conversion of hypoxanthine to uric acid 224 c.mm. The manometric procedure as described under "Enzyme preparation" was employed except when milk was used as the enzyme, then 2.0 ml. of fresh whole milk were employed and no water added.

Davidson (5).[1] Instead of a further oxygen consumption resulting, as would be expected if uric acid was present, a positive pressure was developed in the manometer flask indicating the liberation of a gas. This could not be CO_2, as the center well contained KOH. The most logical explanation would seem to be that the uricase was acting like catalase to break down H_2O_2 which had been formed during the oxidation of hypoxanthine. Dr. Davidson, however, informs me that he has been unable to detect any catalase activity in such uricase preparations. At any rate, there is definitely no oxygen consumption to be observed due to oxidation of uric acid. That the uricase is not inactivated is shown by the fact that the addition of lithium urate produces a rapid oxygen uptake. The conclusion to be drawn would seem to be that no uric acid had been produced by the oxidation of hypoxanthine.

If, however, whole milk instead of a purified sample of xanthine oxidase is used to effect the preliminary oxidation of hypoxanthine, a different result is obtained on the addition of uricase. The curve drawn with a dotted line in Fig. 7 shows such an experiment. Here the addition of uricase produces the expected additional oxygen consumption. Upon the addition of uricase to milk alone no oxygen uptake is observed. A somewhat similar result is obtained when hypoxanthine is oxidized by a purified sample of xanthine oxidase but a crude liver powder is added as a source of uricase. It would thus appear that when purified preparations of both xanthine oxidase and uricase are used some factor is missing that is essential for the conversion of the end-product of hypoxanthine oxidation into a substrate for uricase. Obviously one is led to suggest that of the two forms of uric acid, lactam and lactim, only one is formed by the oxidation of hypoxanthine, and that this one is not a suitable substrate for uricase. The use of a crude preparation as a source of either xanthine oxidase or uricase could then be said to supply some enzyme necessary for the rapid conversion of one form of uric acid into the other. That this is the true explanation of the findings seems doubtful when one considers that such tautomeric changes are usually looked upon as rapid. A decision must await the results of further work.

---

[1] I am indebted to Dr. Davidson for his kindness in supplying me with this preparation.

DISCUSSION

The data given above have been interpreted to indicate that xanthine oxidase is to be classed as a flavoprotein. Since, however, the enzyme has not been crystallized, the objection can be raised that the flavoprotein is an impurity in the preparation. This is of course a possibility, though it seems doubtful since the results indicate strongly that xanthine oxidase activity is closely associated with the pigmented protein. Moreover the flavin content of the purest preparation when reckoned as riboflavin from the absorption coefficient at λ 455 mμ is of the order of 0.5 per cent. This value is in close agreement with the flavin content of the purest preparations of the three other flavoproteins isolated in this laboratory (*cf.* Warburg and Christian (18)). All attempts to purify the enzyme further have so far been unsuccessful. If xanthine oxidase and the flavoprotein are not identical, then the similarity of their isoelectric points and other physical properties will indeed make their separation difficult. Since crystallization of a flavoprotein has yet to be accomplished, the definite proof of the identity or non-identity of xanthine oxidase and the flavoprotein will perhaps best be furnished when the exact chemical nature of the coenzyme of xanthine oxidase is known.

Corran and Green (4) have recently announced the preparation of a flavoprotein from milk. Since their preliminary publication the authors have kindly informed me that they have obtained a preparation containing 0.5 per cent flavin which is devoid of xanthine oxidase activity. Can this flavoprotein be identical with that reported here? Until a full comparison of the properties of the two preparations is possible, this question cannot be answered. The difference in xanthine oxidase activity of the two preparations may of course be explained on the basis of impurity as discussed above. It should be noted, however, that the procedure employed by Corran and Green for the preparation of their flavoprotein involves working at pH 4.0. The flavoprotein preparation described here rapidly loses its xanthine oxidase activity when subjected to such acidities.

SUMMARY

A procedure for obtaining a xanthine oxidase preparation that is 500 times more active per unit of dry weight than the whole milk used as a source is described. Solutions of this enzyme preparation have a strong golden brown color and possess an absorption spectrum with a broad band in the visible region lying between λ 400 and 500 mμ. This band disappears if hypoxanthine is added to a solution from which air is excluded and can be restored by the admission of air. If the spectrum of the reduced enzyme preparation is subtracted from that of the oxidized form, a spectrum is obtained which is similar to that of other yellow enzymes in having two bands centered at λ 370 mμ and λ 465 mμ respectively.

The prosthetic group may be split off from the protein reversibly. Its solutions are pure yellow in color, fluoresce strongly, and possess the characteristic absorption spectrum of a flavin with two bands centered at λ 450 mμ and λ 375 mμ. It can be converted into a lumiflavin derivative identical with that obtained from riboflavin. It functions quantitatively as the coenzyme of the amino acid oxidase and possesses other properties in common with this flavin adenine dinucleotide. The active group of xanthine oxidase is apparently, however, not identical with the amino acid oxidase coenzyme.

The isoelectric point of xanthine oxidase as determined by cataphoresis experiments lies at pH 6.2.

Its maximum molecular weight has been calculated to be 74,000.

The enzyme preparation oxidizes both hypoxanthine and aldehydes but is rapidly destroyed during the oxidation of the latter.

Cyanide irreversibly inhibits its action.

The end-product of hypoxanthine oxidation by purified xanthine oxidase does not act as a substrate for purified uricase. Crude preparations of xanthine oxidase, however, produce an end-product that is oxidized by uricase. The significance of this difference is discussed.

I am indebted to Professor Otto Warburg for his valuable advice and generous provision of laboratory facilities during this investigation.

BIBLIOGRAPHY

1. Ball, E. G., *Science*, **88**, 131 (1938).
2. Booth, V. H., *Biochem. J.*, **29**, 1732 (1935); **32**, 494 (1938).
3. Burian, R., *Z. physiol. Chem.*, **43**, 497 (1905).

4. Corran, H. S., and Green, D. E., *Nature,* **142,** 149 (1938).
5. Davidson, J. N., *Biochem. J.*, **32,** 1386 (1938).
6. Dixon, M., and Keilin, D., *Proc. Roy. Soc. London, Series B,* **119,** 159 (1936).
7. Dixon, M., and Thurlow, S., *Biochem. J.*, **18,** 976 (1924).
8. Haas, E., *Biochem. Z.*, **298,** 378 (1938).
9. Morgan, E. J., Stewart, C. P., and Hopkins, F. G., *Proc. Roy. Soc. London, Series B,* **94,** 109 (1922).
10. Schardinger, F., *Z. Untersuch. Nahrungs- u. Genussmittel,* **5,** 1113 (1902).
11. Spitzer, W., *Arch. ges. Physiol.*, **76,** 192 (1899).
12. Szent-Györgyi, A., *Biochem. Z.*, **173,** 275 (1926).
13. Theorell, H., *Biochem. Z.*, **275,** 1 (1935).
14. Theorell, H., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **6,** 111 (1937).
15. Warburg, O., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **7,** 210 (1938).
16. Warburg, O., and Christian, W., *Biochem. Z.*, **266,** 377 (1933).
17. Warburg, O., and Christian, W., *Biochem. Z.*, **298,** 150 (1938).
18. Warburg, O., and Christian, W., *Biochem. Z.*, **298,** 368 (1938).

PROTEIN ANABOLISM OF ORGANS AND TISSUES DURING PREGNANCY AND LACTATION*

BY L. J. POO, W. LEW, AND T. ADDIS

(From the Department of Medicine, Stanford University Medical School, San Francisco)

(Received for publication, December 20, 1938)

It has been demonstrated that during a fast the various organs and tissues of the body lose protein at very different rates, some rapidly, others slowly, and others not at all (1). A similar disparity was found in the rate of gain in the protein content of different organs on refeeding after a fast (2). The significance of these differences in the various organs is not always obvious and this is especially true in the case of the liver, the organ in which the greatest rate and degree of change were observed. One of the reasons that led us to measure the total protein and organ protein content of pregnant and lactating rats was the hope that further information might be obtained as to the rôle of the liver in protein metabolism, but the principal incentive was the fact that gestation and lactation provide an opportunity to observe the effect of a drastic alteration in the conditions under which the processes of protein metabolism are ordinarily conducted. During pregnancy the formation of the embryos draws from the maternal blood the materials required for protein formation, while at the same time the enlargement of the uterus and formation of the placentas require a considerable preponderance of protein anabolism over catabolism. During lactation the whole of the rapidly increasing protein content of the litter is transferred through the milk proteins from the mother to the young. If in the non-pregnant, non-lactating female rats we find what part of the total protein of the body is allocated to the heart, kidney, liver, carcass, etc., we shall be able on comparing this control

---

* This work was aided by a grant from the Rockefeller Foundation.

distribution with the distribution in the bodies of pregnant and lactating rats to answer the question as to what changes in protein distribution have occurred in the maternal body under these quite singular conditions.

100 female rats from 90 to 100 days of age were divided into five groups of twenty rats each in such a manner that the average body weight per rat in each group was 150 gm. on the day on which a diet containing 16 per cent of protein with adequate vitamin and mineral content was started.[1] One group was sacrificed at once, another after 18 days, and another 31 days after the diet was begun. These were the three control groups and the increase in protein content over the period of observation measured the growth of protein in non-pregnant females. There were two experimental groups. From a number of females that had been kept with males for the previous 5 days two groups of twenty pregnant rats were chosen, each of which had an average body weight of 150 gm. when started on the 16 per cent protein diet. The rats of one of these groups were killed 18 days later when, on the average, they were at the 20th day of pregnancy. Those of the other groups went to term and were killed on the 31st day when, on the average, they had been giving milk to their young for 10 days. Since both the litters of the lactating rats and the embryos of the pregnant rats had grown solely on food derived from their mothers, their protein content was determined and added to the maternal protein. The total body and organ weights are given in Table I and the corresponding protein quantities in Table II.

---

[1] The diet contained 16 per cent of casein, 9 per cent of yeast, 2 per cent of alfalfa, 10 per cent of sardine oil, 15 per cent of lard, 44 per cent of cornstarch, and 4 per cent of the Osborne-Mendel salt mixture (3). An attempt was made to determine the protein content of the air-dry casein and yeast. The total nitrogen concentrations were 13.26 and 8.39 per cent and the non-protein nitrogen concentrations 0.32 and 2.91 per cent respectively. Three methods for precipitating the protein, washing, extracting the fat, and drying were used and the estimates made either by direct weighings or by nitrogen determinations. We were not satisfied that any of these methods gave entirely reliable results but on averaging all our observations we concluded that the best estimate for the protein content of the air-dry casein was 79.1 per cent. In the case of the yeast the average protein content was found to be 46.0 per cent but consideration of the availability as food led us to reduce this estimate to 31.3 per cent. According to these figures the protein content of the diet is 15.86 per cent.

TABLE I

Average Weight in Gm. per Rat of Organs and Tissues of Pregnant, Lactating, and Control Groups of Twenty Rats with an Average Body Weight of 150 Gm. at 0 Days

	Initial control at 0 days	Pregnancy at 18 days	Control for pregnancy at 18 days	Lactation at 31 days	Control for lactation at 31 days
Body weight of mothers....	150	204	172	162	179
Weight of young...........		27.057		74.900	
Total weight..............	150	231.057	172	236.900	179
Uterus....................	0.290	10.768	0.383	(0.326)	0.387
Alimentary tract, etc.*.....	34.751	30.965	25.312	28.037	26.708
Drawn blood,† serum......	2.876	3.450	2.935	3.040	2.876
" " clot........	2.452	2.545	2.550	2.465	2.452
Heart....................	0.519	0.623	0.573	0.582	0.597
Kidney....................	0.995	1.114	1.076	1.055	1.089
Liver.....................	6.567	8.835	6.271	7.745	7.391
Carcass‡.................	101.550	145.700	132.900	118.750	137.500

* The alimentary tract, etc., includes the entire abdominal and pelvic contents, except the kidneys, liver, and uterus. All visible abdominal and pelvic fat was included.

† Drawn blood is all the blood that could be obtained from the cut abdominal aorta of the anesthetized rat while the heart was still beating. After centrifuging at high speed the serum and clot were separated and weighed.

‡ The carcass is the whole rat after exsanguination, with the heart excised, stripped of all abdominal and pelvic organs and all visible fat.

TABLE II

Average Protein Content in Gm. per Rat of Organs and Tissues of Pregnant, Lactating, and Control Groups of Twenty Rats with an Average Body Weight of 150 Gm. at 0 Days

	Initial control at 0 days	Pregnancy at 18 days	Control for pregnancy at 18 days	Lactation at 31 days	Control for lactation at 31 days
Protein in mothers.........	23.546	29.446	27.325	26.525	29.573
" " young..........		2.072		9.429	
Total protein..............	23.546	31.518	27.325	35.954	29.573
Maternal protein					
Uterus................	0.040	0.822	0.049	(0.051)	0.055
Alimentary tract, etc....	1.631	1.809	1.447	1.858	1.685
Drawn blood, serum.....	0.166	0.185	0.196	0.140	0.185
" " clot.......	0.712	0.714	0.743	0.668	0.804
Heart..................	0.083	0.103	0.098	0.097	0.101
Kidney.................	0.161	0.177	0.177	0.173	0.183
Liver..................	1.256	1.602	1.194	1.330	1.393
Carcass................	19.497	24.034	23.421	22.208	25.167

Since at the beginning all the groups were identical, we can subtract the 23.5 gm. of total protein they then contained from the quantities found 18 and 31 days later. In the control groups the increase is the new protein formed during growth and in the pregnant and lactating groups the added protein is the combined result of maternal growth and the growth of the young. Table II shows that in the controls there was an increase of 3.8 gm. of protein in 18 days and of 6.0 gm. in 31 days, while in the pregnant groups the increase was 8.0 gm. and in the lactating groups 12.4 gm. of protein. Pregnancy alone is therefore responsible for the formation of 4.2 gm. of new protein, while on the 10th day of lactation 6.4 gm. of protein more than the amount to be anticipated from maternal growth had been laid down. Under both conditions the rate of protein anabolism is more than doubled.

It was not possible to get reliable food measurements in the lactating group but in the pregnancy group the total protein taken in the food during 18 days was 34.8 gm. per rat and, since there was a new formation of 8 gm. of protein, 23 per cent of the protein eaten was used for anabolism. In the corresponding control group 24.2 gm. per rat were eaten and of this amount 16 per cent was used in growth.

A comparison of Tables I and II shows that the gain in weight during pregnancy and lactation is out of proportion to the gain in protein, so that the concentration of total protein per 100 gm. of total weight falls during pregnancy and lactation relatively to the controls. This is largely due to the high water content of the uterine contents in pregnancy (the embryos contained 7.7 per cent of protein) and of the 10 day-old litters (the protein concentration of the litters was 12.6 per cent). But this is not the only reason, for, if we consider the protein concentration in the organs and tissues of the mother alone, it will be noted that with the exception of the alimentary tract, and the carcass in the lactating group, there is a general reduction in protein per 100 gm. of organ or tissue weight. The order and extent of this protein dilution are shown in Figs. 1 and 2.

This reduction in protein concentration cannot be ascribed to an increase in the fat content of the organs and tissues in pregnancy and lactation. Inspection suggested a decrease of fat content and this is borne out by the fact that the alimentary

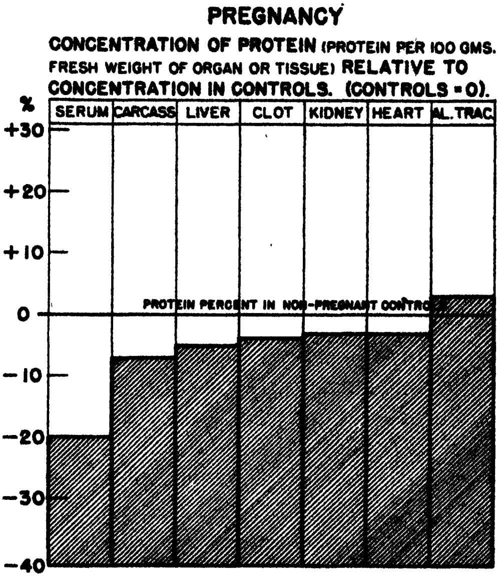

PREGNANCY
CONCENTRATION OF PROTEIN (PROTEIN PER 100 GMS. FRESH WEIGHT OF ORGAN OR TISSUE) RELATIVE TO CONCENTRATION IN CONTROLS. (CONTROLS = 0).
%
SERUM
CARCASS
LIVER
CLOT
KIDNEY
HEART
AL. TRAC.
+30
+20
+10
0
-10
-20
-30
-40
PROTEIN PERCENT IN NON-PREGNANT CONTROLS

Fig. 1

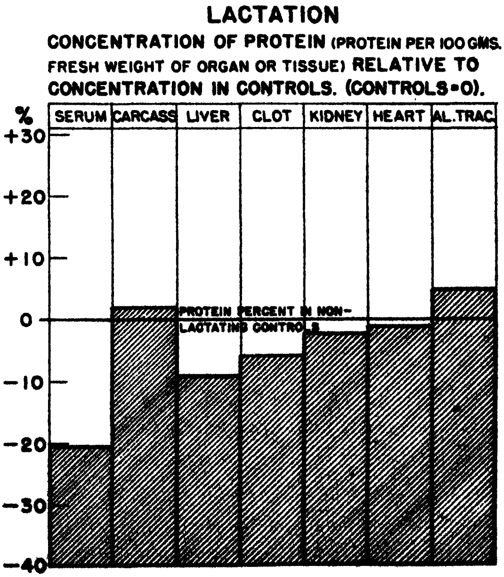
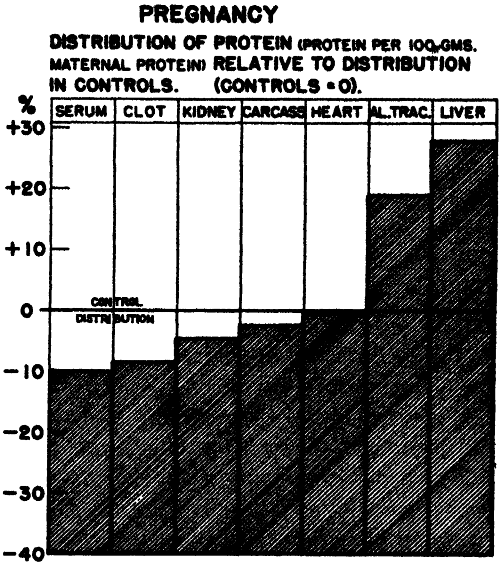

LACTATION
CONCENTRATION OF PROTEIN (PROTEIN PER 100 GMS. FRESH WEIGHT OF ORGAN OR TISSUE) RELATIVE TO CONCENTRATION IN CONTROLS. (CONTROLS = 0).
%
SERUM
CARCASS
LIVER
CLOT
KIDNEY
HEART
AL. TRAC.
+30
+20
+10
0
-10
-20
-30
-40
PROTEIN PERCENT IN NON-LACTATING CONTROLS

Fig. 2

tract in whose weight all visible abdominal and pelvic fat is included is an exception to the general rule and has a higher concentration of protein than the controls. It is therefore highly probable that the reduction in protein concentration is due to an increased water content of the maternal tissues, though it cannot be concluded that this is a phenomenon always characteristic of pregnancy and lactation because our observations were made under one set of dietary conditions, and the food given contained only 16 per cent of protein, almost all in the form of casein.

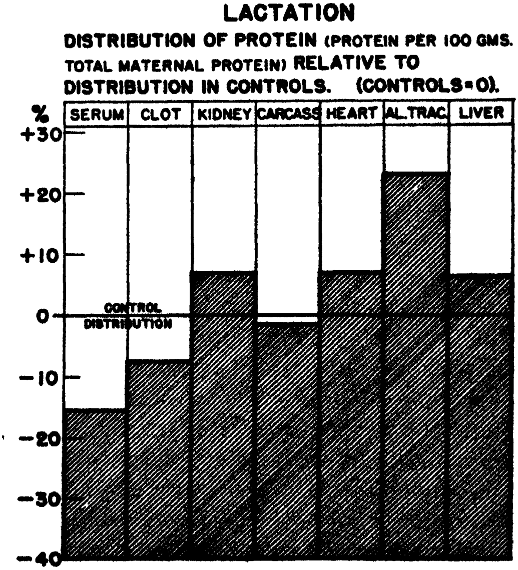
The concentration of protein in the organs and tissues is in itself a measurement whose significance is ambiguous, since it may be altered by changes in either water or fat content. There is no such doubt when we consider the total protein content of the body and find what proportion of that total is allocated to the various organs and tissues, for protein is the essential operating machinery of the body and the proportion distributed to each organ can be regarded as an indication of its significance in the total economy. Our reason for this statement rests on the observation that when conditions are devised that increase or decrease the work of the heart or kidney we have found a corresponding rise or fall in the proportion of the total protein found in these organs, so that if time for adjustment is allowed the pattern of distribution of protein in the various parts of the body may be taken as an indicator of function. During pregnancy and lactation, on the diet we gave, the conditions with respect to protein are of such a nature as to necessitate an unusual economy in the distribution of the total available amount. During pregnancy the maternal organism becomes subsidiary to the needs of the rapidly growing parasitic embryos, and throughout lactation the drain on the maternal resources is continued through the provision in the milk of all of the protein used by the young. Under such conditions deviations from the distribution in the organs and tissues of control rats subjected to no such drain are of interest as a rough indication of their relative functional importance. This distribution of maternal protein (total protein less the protein of the uterus, the embryos, and the young) in pregnancy and lactation relative to that found in the controls is shown in Figs. 3 and 4.

The results show that in pregnancy the serum and clot of the drawn blood, the kidney, and the carcass have smaller proportions



PREGNANCY
DISTRIBUTION OF PROTEIN (PROTEIN PER 100 GMS. MATERNAL PROTEIN) RELATIVE TO DISTRIBUTION IN CONTROLS. (CONTROLS = 0).

Fig. 3



LACTATION
DISTRIBUTION OF PROTEIN (PROTEIN PER 100 GMS. TOTAL MATERNAL PROTEIN) RELATIVE TO DISTRIBUTION IN CONTROLS. (CONTROLS = 0).

Fig. 4

of the total protein assigned to them and that other organs, the alimentary tract and the liver, have considerably more than the controls. The increase in the alimentary tract may be a result of the work hypertrophy that has been shown to follow an increase in the bulk of food consumed (4). Whether the decrease in serum, clot, and carcass protein is a depletion effect arising because the gastrointestinal tract was mechanically incapable of carrying the quantity of food containing only 16 per cent of protein that was required to meet the increased demand is a question that can be answered by observing the effect of foods with higher protein concentrations. If with increase in total protein consumption we no longer find any decrease in the amount of protein allocated to these tissues, the simple depletion hypothesis is validated. But if this decrease is found even when more than adequate quantities of protein are taken, we must look for some other mechanism. These considerations apply also to the decrease in serum, clot, and carcass protein proportions during lactation, while the increase in the kidney protein in lactation suggests a greater consumption and catabolism of protein, since in other experiments we have found that the proportion of protein in the kidney varies with nitrogen excretion.

The unexpected and most interesting result is the 28 per cent increase in the amount of protein allocated to the liver in pregnancy, unexpected because in other experiments in which the effect of dietetic or metabolic changes has been observed there has been agreement in the direction of change in kidney and liver protein changes (5) and interesting because the reason for the increase in liver protein is uncertain. One possibility is that it represents a store of protein laid down against the need of protein for milk formation, and that this storage is a special adaptation for the preservation of the species developed in rodents under conditions of life in which a temporary shortage of protein may be a frequently recurring event during lactation. But if the decrease in blood and carcass protein is the effect of a depletion of protein, this storage must be a very special adaptation, since as a rule there is a very rapid decrease in liver protein whenever the protein intake is inadequate (6). The other possibility is that the increase is the result of a work hypertrophy of the liver during pregnancy. But we have no knowledge as to the nature of this hypothetical work. At best we can relate it to the increase in anabolic activity in pregnancy and suppose that it may be some energy-requiring preparation of material utilized by the embryos. As between storage or work hypertrophy it may be hoped that total protein and organ protein determinations on pregnant rats given protein quantities from bare maintenance to surplus amounts will be decisive. These observations are now being made.

SUMMARY

1. The rate of protein anabolism in growing rats is more than doubled towards the end of pregnancy and on the 10th day of lactation.

2. The concentration of protein per 100 gm. of organ or tissue weight is most reduced in the serum and in successively less degree in the liver, blood clot, kidney, and heart in both pregnancy and lactation on a diet that contained 16 per cent of protein.

3. The proportion of the total maternal protein allocated to the serum, blood clot, kidney, and carcass is reduced in pregnancy. In lactation the serum, blood clot, and to a slight degree the carcass have less than the usual proportions of the total protein. In both pregnancy and lactation the alimentary tract contains more than the usual proportion of protein.

4. There is a 28 per cent increase in the proportion of protein assigned to the liver in pregnancy.

BIBLIOGRAPHY

1. Addis, T., Poo, L. J., and Lew, W., *J. Biol. Chem.*, **115**, 111 (1936).
2. Addis, T., Poo, L. J., and Lew, W., *J. Biol. Chem.*, **116**, 343 (1936).
3. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
4. Addis, T., *Am. J. Physiol.*, **99**, 417 (1932).
5. Walter, F., and Addis, T., *J. Exp. Med.*, **69**, 467 (1939).
6. Addis, T., Poo, L. J., and Lew, W., *J. Biol. Chem.*, **115**, 117 (1936).

NOTE ON THE ACTION OF MANGANESE AND SOME OTHER METALS ON THE OXIDATION OF CERTAIN SUBSTANCES BY LIVER

BY FREDERICK BERNHEIM AND MARY L. C. BERNHEIM

(From the Departments of Physiology and Pharmacology and Biochemistry, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, January 6, 1939)

Titanium with an atomic number of 22 and vanadium with an atomic number of 23 have been shown to have specific actions on certain biological oxidations (1). It was therefore of interest to study the effects of chromium, manganese, iron, cobalt, and nickel, elements with atomic numbers from 24 to 28. Addition of these elements in the form of various salts to rat liver suspensions showed that, with the exception of manganese, they had very little effect on the oxygen uptake. Manganese in concentrations of M/20,000 caused a definite inhibition of the oxygen uptake of rat liver, but not of kidney and brain. This inhibition was greatest at pH 6.7 and fell off rapidly with increasing alkalinity. The oxidation of amino acids, amines, uric acid, choline, alcohol, lactate, succinate, and glucose was not affected by the presence of manganese. The oxidation of phospholipids by the vanadium-protein system and the oxidation of cysteine were definitely inhibited.

EXPERIMENTAL

The rat liver was prepared by the method already described (1) and the procedure outlined in these two papers was followed exactly. For the phospholipid an alcohol extract of rat liver was used. Fig. 1 shows the effect of the various salts on the oxidation of phospholipids catalyzed by the vanadium-protein system. The salts were dissolved in water and added to the liver suspension. Manganese inhibits the oxidation completely, cobalt partially, and none of the other elements has any effect. To this negative group belong titanium (6 micrograms) added as sodium pertitanate and chromium (5 micrograms) added as chromium potassium sulfate or potassium dichromate. Higher concentrations of

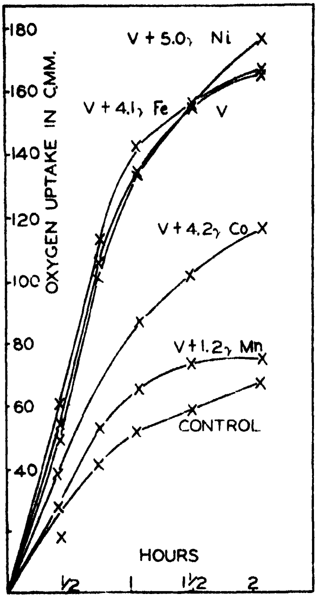


FIG. 1

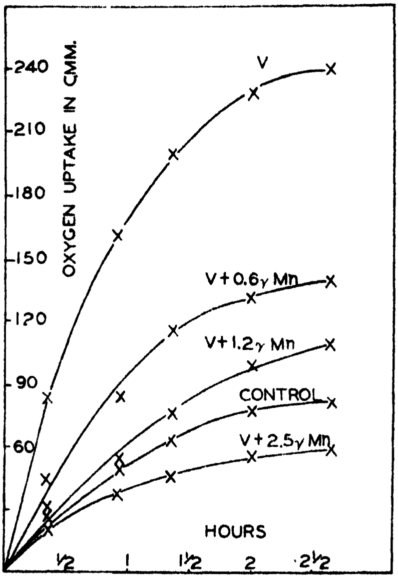


FIG. 2

FIG. 1. The effect of various metals on the oxidation of rat liver phospholipid in the presence of 0.5 cc. of washed rat liver protein and 0.1 mg. of sodium metavanadate at pH 6.7 and 37°. Beside nickel chloride and ferric chloride, 5 micrograms of chromium as chromium potassium sulfate or potassium dichromate and 6 micrograms of titanium as sodium pertitanate are without effect. The cobalt was added as cobalt sulfate, the manganese as manganese chloride. The volume in each vessel was 2.0 cc. The control represents the uptake of protein and phospholipid without vanadium. The uptake of the protein and various metals has been subtracted from the values of the curves which represent the oxidation of the phospholipid.

FIG. 2. The effect of various concentrations of manganese as manganese chloride on the oxidation of rat liver phospholipids by the vanadium-protein system at pH 6.7 and 37°. Manganese added as potassium permanganate gives similar concentration curves. The volume in each vessel was 2.0 cc. The uptake of protein and metals without phospholipid was substracted from the values of each curve. The control represents the uptake of protein and phospholipid without vanadium.

these elements show some inhibition. Fig. 2 shows the effect of different concentrations of manganese on the vanadium-catalyzed phospholipid oxidation. Exactly similar curves can be obtained if the manganese is added as potassium permanganate. Manganese also inhibits the slow oxidation of phospholipid by the washed liver protein in the absence of vanadium.

Titanium has been shown to inhibit the oxidation of cysteine to its sulfonic acid. Table I shows the effect of the various other metals on this oxidation. Manganese and cobalt show complete inhibitions in very small concentrations. Chromium shows some inhibition but vanadium and nickel are without effect. Increasing the concentrations 10 times causes marked inhibitions in all cases except vanadium. The metals have a similiar effect on the oxidation of thioglycolic acid.

TABLE I

Effect of Manganese Chloride, Cobalt Sulfate, Nickel Chloride, Potassium Dichromate, Chromium Potassium Sulfate, and Sodium Metavanadate on Oxygen Uptake of 0.25 Mg. of Cysteine Hydrochloride (Neutralized) in Presence of 0.5 Cc. of Washed Liver Protein at pH 6.7 and 37°

The figures given in micrograms are the amounts of metal present in the salts added. The total volume of fluid in each vessel was 2.0 cc.

Time	Alone	2.5 micrograms Mn	2.2 micrograms Co	2.5 micrograms Ni	3.5 micrograms Cr, ($K_2Cr_2O_7$)	3.0 micrograms Cr, ($Cr_2K_2(SO_4)_4$)	20.0 micrograms V
min.	*c.mm. O_2*	*c.mm. O_2*	*c.mm. O_2*	*c.mm. O_2*	*c.mm. O_2*	*c.mm. O_2*	*c.mm. O_2*
15	25	2	2	27	5	15	26
30	41	6	5	41	14	20	44
45	51	8	7	45	25	25	53
60	54	8	8	51	29	28	55
90	56	6	7	56	37	29	57
135	55	4	6	58	39	30	53
155	53	4	4	59	42	32	54

DISCUSSION

As manganese (2) as well as vanadium is found in all livers, it is possible that these two elements acting in different directions on phospholipid oxidation may be used by the cell to regulate the rate of the oxidation.

SUMMARY

1. Manganese and, to a lesser extent, cobalt inhibit the oxidation of phospholipid by the washed liver protein-vanadium system. Nickel, iron, titanium, and chromium have no effect.

2. Manganese and cobalt as well as titanium inhibit the oxidation by washed liver protein of cysteine to its sulfonic acid.

BIBLIOGRAPHY

1. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **127**, 353, 695 (1939).
2. Dubuisson, M., and Thomas, F., *Ann. physiol. et physicochim. biol.*, **5**, 857 (1929).

CHEMICAL DIFFERENCES OF THE FOLLICLE-STIMULATING AND LUTEINIZING HORMONES OF THE PITUITARY*

BY H. L. FEVOLD

(*From the Biological Laboratories, Harvard University, Cambridge*)

(Received for publication, January 11, 1939)

The follicle-stimulating and the luteinizing hormones of the anterior pituitary have been separated and characterized physiologically as two distinct gonadotropic principles (1, 2). Chemically, however, the two factors have thus far been differentiated only by their individual solubilities in aqueous solvents, the luteinizing hormone being less soluble and more easily precipitated than the follicle-stimulating hormone. Recently, McShan and Meyer (3) have reported that the follicle-stimulating hormone differs from the luteinizing hormone by being destroyed by ptyalin but not by trypsin, whereas the reverse is true for the luteinizing hormone.

This paper reports certain reactions which demonstrate a true chemical difference between the two hormones. When the two hormones are treated with picrolonic, flavianic, or picric acid, precipitation of both substances is complete, but whereas the follicle-stimulating hormone is inactivated, the luteinizing hormone retains its physiological activity. Furthermore, the inactivation of the follicle-stimulating hormone is reversible and the activity is regenerated when the acid is removed.

A further difference in the two hormones is shown by intraperitoneal injection. The luteinizing hormone is fully as active when given by this route as when injected subcutaneously, while the follicle-stimulating hormone is almost completely ineffective when injected into the peritoneal cavity.

---

* Aided by a grant from the Rockefeller Foundation, administered by Frederick L. Hisaw.

EXPERIMENTAL

Material and Methods

The follicle-stimulating and the luteinizing hormones used in these experiments were taken from water-soluble stock solutions prepared from fresh sheep pituitaries. For standardization and injection, both were routinely precipitated with tannic acid and injected as insoluble tannates. It has been shown previously that tannic acid exerts no other effect on the hormones than to make them insoluble, thereby decreasing the rate of absorption and consequently increasing the efficiency of utilization (4).

The animals which were used in these experiments were 21 day-old male and female rats. The tests on the females were carried out by injecting the preparation in 0.25 cc. doses twice daily for 3 days and weighing the ovaries the morning of the 4th day. The males were similarly injected for 5 days and the seminal vesicles plus coagulation glands weighed the morning of the 6th day. The figures reported in Tables I to VI are the results of final experiments and are the averages of the weights of the glands of at least three animals in each instance.

Effect of Picrolonic, Flavianic, and Picric Acids on the Follicle-Stimulating, Luteinizing, and Unfractionated Extracts (Follicle-Stimulating Plus Luteinizing Hormones)—1 cc. of the aqueous solution of the follicle-stimulating hormone, containing 80 rat units per cc., was treated, drop by drop, with an acetone solution of picrolonic acid until precipitation was complete. The precipitate was washed once with 2 cc. of distilled water, centrifuged, and emulsified in water. One-half of this preparation was used for testing in immature rats. The remainder, in 3 cc. of water, was just dissolved by the careful addition of 10 per cent NaOH, the pH after solution being approximately 6.5. The dissolved material was precipitated by being poured into 30 cc. of cold acetone; the precipitate was collected by centrifuging, dissolved in 3 cc. of water, precipitated with tannic acid, and prepared for injection as before. The same procedure was followed in making picric acid and flavianic acid precipitates, except that aqueous solutions of these acids were used.

The results (Table I) clearly show that the precipitates of the follicle-stimulating hormone with picrolonic, picric, and flavianic acids were inactive in promoting follicular growth. The highest dosages of the picric and flavianic acid precipitates produced slight increases in ovarian weight (less than one-sixth of the original potency) but the picrolonic acid precipitate was completely ineffective, even at the highest dose. However, after the acids were removed as described, the follicle-stimulating hormone was as active as before. It is evident therefore that the acids combined with the active substance in such a way as to render the prosthetic group inactive, the prosthetic group being again set free when the acid was removed.

A purified preparation of luteinizing hormone, free of follicle-stimulating hormone and containing 40 male units per cc., was prepared for injection by precipitation with tannic, picrolonic, picric, and flavianic acids in the same manner as described for the follicle-stimulating hormone. Upon assay, all the precipitates were found to be of approximately equal potency, showing no indications of inactivation (Table II).

TABLE I

Effect of Picrolonic, Picric, and Flavianic Acids on Follicle-Stimulating Hormone

Dosage, *rat units*	6	3	1
	Ovarian weight, mg.		
Tannate	58	38	22
Picrolonate	13	13	12
Picrate	18	13	11
Flavianate	17	15	12
Picrolonate to tannate	51	33	23
Picrate to tannate	73	35	20
Flavianate to tannate	71	29	21
Free aqueous follicle-stimulating hormone	35	28	17

A crude unfractionated extract of fresh sheep pituitary tissue was prepared in three forms: (1) tannate, (2) picrolonate, (3) picrolonate which was converted to tannate. All of these preparations were tested in male and female immature rats. The results show that the activity in immature females was lost on precipitation with picrolonic acid, but was regenerated when the material was again freed from the acid. In the male a difference between the response to the tannate and to the picrolonate is noted. With the picrolonate, a limit was quickly reached at 20 to 25 mg. and no further increase in seminal vesicle response took place with increase of dosage. Equivalent amounts of the tannic acid precipitate gave a greater response than the picrolonate, the seminal vesicles continuing to increase with increasing dosage of the tannate up to 50 mg. (Table III).

TABLE II

Non-Effect of Picrolonic, Picric, and Flavianic Acids on Luteinizing Hormone

Dosage, *rat units*	0.5	1.0	2.0	4.0	8.0	16.0
	Weight of seminal vesicles, mg.					
Tannate	14	18	18	20	21.5	23
Picrolonate	13	18	19	19	20	21
Picrate	15	19	19	22	21	22
Flavianate	13	18	20	19	23	24

TABLE III

Effect of Picrolonic Acid on Unfractionated Anterior Pituitary Gonadotropic Extract

		1.0	0.5	0.25	0.1	0.05
		Weight of ovaries or seminal vesicles, mg.				
Tannate	♀	110	85	60	43	32
Picrolonate	♀	12	13			
" to tannate	♀	98	87	47	40	36
Tannate	♂	50	43	37	30	26
Picrolonate	♂	23	23	21	22	19
" to tannate	♂	52	50	40	29	27

* Dosages are given in gm. equivalents of fresh tissue.

We have previously reported that while the follicle-stimulating hormone alone does not cause secretion of male hormone it does increase the action of the luteinizing hormone in producing secretion of androgen, as measured by the weight of the seminal vesicles (5). Since picrolonic acid inactivates the follicle-stimulating hormone, it appears that the difference between the responses to the material precipitated with tannic acid and to the same material precipitated with picrolonic acid is due to the presence of active follicle-stimulating hormone in one preparation and not in the other. Further evidence showing that this is so is obtained from a comparison of intraperitoneal and subcutaneous injections of unfractionated extracts.

Relative Efficiency of Subcutaneous and Intraperitoneal Injections of Tannic and Picrolonic Acid Preparations of Luteinizing, Follicle-Stimulating, and Follicle-Stimulating Plus Luteinizing Hormones—In order to test the comparative efficiency of subcutaneous and intraperitoneal injections of luteinizing hormone, it was precipitated with tannic acid and also with picrolonic acid and each preparation was injected in two groups of immature male rats, one group being injected subcutaneously and the other intraperitoneally. The results show that both preparations were equally effective by either method of injection (Table IV).

Table IV

Comparative Efficiency of Subcutaneous and Intraperitoneal Injections of Luteinizing Hormone

Dosage, *rat units*	0.5	1.0	2.0	4.0	8.0	16.0
	Weight of seminal vesicles, mg.					
Tannate, subcutaneous	15	19	21	21	24	23
" intraperitoneal	13	17	22	20	22	21
Picrolonate, subcutaneous	16	18	21	20	23	21
" intraperitoneal	17	19	18	22	21	22

Intraperitoneal injections of follicle-stimulating hormone were ineffective as compared to subcutaneous injections. Slight stimulation was obtained with 5 and 15 rat units, as shown by some uterine enlargement. The rats given 1 unit showed no stimulation, as judged by either ovarian or uterine response. The ovarian weights after the subcutaneous injection of 1, 5, and 15 rat units were 22, 44, and 62 mg. respectively; after corresponding intraperitoneal injections, 10, 18, and 15 mg.

The effect of follicle-stimulating hormone on the action of luteinizing hormone was further evidenced when the picrolonic and tannic acid precipitates of an unfractionated gonadotropic extract were injected in immature males, subcutaneously and intraperitoneally. As before, the picrolonate was just as effective in causing hypertrophy of the seminal vesicles when injected intraperitoneally as it was subcutaneously. The material precipitated with tannic acid, however, showed the same activity as the picrolonate when injected intraperitoneally, but was more effective when injected subcutaneously (Table V). The explanation of this becomes evident when we consider that the follicle-stimulating hormone is not effective when injected intraperitoneally, and that it is likewise inactivated by picrolonic acid. The results obtained when the picrolonic acid preparation is injected either subcutaneously or intraperitoneally or when the material precipitated with tannic acid is injected intraperitoneally are due to the action of luteinizing hormone alone on the interstitial elements. When the tannic acid preparation is injected subcutaneously, the results reflect the action of the luteinizing hormone augmented by the follicle-stimulating hormone which is present and is, under these conditions, free to act.

TABLE V

Subcutaneous and Intraperitoneal Injection of Tannic and Picrolonic Acid Precipitates of Unfractionated Extract in Immature Rats

Dosage*	1.0	0.5	0.25	0.1	0.05
	Weight of seminal vesicles, mg.				
Tannate, subcutaneous	48	43	36	28	20
" intraperitoneal	23	23	22	20	18
Picrolonate, subcutaneous	24	22	20	19	19
" intraperitoneal	25	22	20	18	19

* Dosages are given in gm. equivalents of fresh tissue.

Effect of Luteinizing, Follicle-Stimulating, and Follicle-Stimulating Plus Luteinizing Hormones on Male Hormone Secretion in Hypophysectomized Rats—Since the ability of the luteinizing hormone to produce secretion of male hormone was so sharply limited in normal immature rats, a series of hypophysectomized rats was treated with luteinizing hormone to determine whether this was still more markedly the case when the animal's own pituitary was unable to contribute to the reaction. The rats were hypophysectomized when they were 28 days old, and a postoperative period of 7 days allowed to elapse before injections were started. Three groups of rats received 2, 8, and 16 rat units respectively, over a period of 8 days. Another group received 8 units of follicle-stimulating hormone, while still another received 8 units of this hormone plus 8 units of luteinizing hormone. In none of the rats receiving the luteinizing hormone alone did the testes descend into the scrotum, whereas the testes of those receiving both hormones assumed the scrotal position after 3 or 4 days of treatment. The seminal vesicles of the rats receiving follicle-stimulating hormone alone were not enlarged over those of the controls and the enlargement produced with luteinizing hormone alone was not very great and was not markedly different in those receiving 2, 8, or 16 units. In contrast, the seminal vesicles of those receiving follicle-stimulating in addition to luteinizing hormone were much more enlarged, showing definitely the complementary effect of the follicle-stimulating hormone (Table VI).

TABLE VI

Comparative Effect of Follicle-Stimulating, Luteinizing, and Follicle-Stimulating Plus Luteinizing Hormones on Seminal Vesicles of Hypophysectomized Rats

	Dosage	Seminal vesicles	Testes
	rat units	*mg.*	*mg.*
Follicle-stimulating	8	6	170
Luteinizing	16	12	152
"	8	13	150
"	2	11	138
" + follicle-stimulating	8 + 8	37	350
Controls	0	5	82

DISCUSSION

The difference in the reaction of the follicle-stimulating and luteinizing hormones to the reagents used in this investigation indicates that the prosthetic groups responsible for follicle stimulation and luteinization must be different. The active group of the follicle-stimulating hormone apparently combines with the reagents, thereby becoming inactive, whereas that of the luteinizing hormone either does not so combine or at least is not inactivated by the combination. A basic group is indicated as being necessary for the physiological activity of the follicle-stimulating hormone, while that responsible for luteinizing activity is of a different type.

The intimate association of these two factors in gonadal development in the male is demonstrated by the facts which have been presented. It is apparent that the action of luteinizing hormone on the interstitial elements of the testes is enhanced by the presence of follicle-stimulating hormone. While the latter hormone alone does not elicit secretion from the interstitial cells, its action on the testes increases the stimulation of the interstitial cells by the luteinizing hormone. It is apparent that in a 21 to 26 day-old rat, or in hypophysectomized rats, the amount of secretion of male hormone from the interstitial cells, as indicated by seminal vesicle hypertrophy, which can be induced by luteinizing hormone alone, is limited. When follicle-stimulating hormone is present, however, the secretion of male hormone is increased and the seminal vesicles are very much enlarged.

Since these two hormones apparently act on different structures of the testes, namely the interstitial cells and the tubules, it is difficult to understand their synergism. The follicle-stimulating and luteinizing hormones act on the same general structure in the female gonad, so one can readily understand that one factor conditions the structure for the other. In the male, however, the luteinizing hormone has no direct spermatogenic effect on the tubules and the follicle-stimulating hormone does not stimulate interstitial cell hypertrophy and secretion of male hormone, but it does increase male hormone secretion when present with the luteinizing hormone. Whether it does this directly or indirectly through the tubules cannot be answered at the present time.

It has been generally believed that gonadotropic extracts of the pituitary are ineffective when given by the intraperitoneal route. This belief was based on the fact that pituitary extracts, so injected into immature female rats, failed to induce ovarian hypertrophy. It is now clear that this is due to a failure of the action of follicle-stimulating hormone, for without it luteinizing hormone does not produce gross effects on the ovary. Luteinizing hormone is equally effective by either subcutaneous or intraperitoneal route, as demonstrated in immature male rats. If an unfractionated extract is injected intraperitoneally in immature males, the response obtained is much less than if it is given subcutaneously. This can be explained by the fact that the follicle-stimulating hormone is not effective and the augmentation effect between the follicle-stimulating and the luteinizing hormone is not obtained. The results represent the effect of the luteinizing hormone acting alone and are equal in magnitude to those obtained when the follicle-stimulating hormone of the same preparation is inactivated by precipitation with picrolonic acid.

Heretofore the difficulty of assaying unfractionated extracts for either of the two pituitary gonadotropic hormones (follicle-stimulating and luteinizing) has been that the interaction between the two in any physiological reaction gives results which are not quantitative for either. The most convenient method for standardizing the luteinizing hormone takes advantage of its property of stimulating the interstitial tissue of the male gonad to secrete male hormone with consequent enlargement of the secondary sex glands. By determining the increase in the weight of the seminal vesicles it is possible to establish a unit. However, in the presence of follicle-stimulating hormone, the action of a given amount of luteinizing hormone is increased, so that the effect is partially due to the former. This difficulty can now be obviated and the true value of the luteinizing hormone determined in two ways: either by injecting the material intraperitoneally, or by precipitating the material with picrolonic acid and assaying the precipitate. The unit is the same as previously defined; namely, the smallest amount necessary to produce a 100 per cent increase in the weight of the seminal vesicles (1).

SUMMARY

1. The follicle-stimulating hormone is precipitated and inactivated by picrolonic, picric, and flavianic acids. The reaction is reversible and the activity is regenerated when the acids are removed from the precipitates.
2. The luteinizing hormone is not inactivated by these reagents.
3. The luteinizing hormone is as effective when injected intraperitoneally as when injected subcutaneously.
4. The follicle-stimulating hormone is ineffective when injected into the peritoneal cavity.
5. The follicle-stimulating hormone, while itself incapable of stimulating the interstitial cells of the testes to secrete male hormone, augments the action of the luteinizing hormone in this reaction.

BIBLIOGRAPHY

1. Fevold, H. L., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **5**, 93 (1937).
2. Hisaw, F. L., Fevold, H. L., and Greep, R. O., *Proc. Assn. Research Nerv. and Ment. Dis.*, **17**, 246 (1936).
3. McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, **126**, 361 (1938).
4. Fevold, H. L., Hisaw, F. L., Hellbaum, A., and Hertz, R., *Am. J. Physiol.*, **104**, 710 (1933).
5. Greep, R. O., Fevold, H. L., and Hisaw, F. L., *Anat. Rec.*, **65**, 261 (1936).

THE CYSTINE CONTENT OF DEAMINIZED PROTEINS*

By W. C. HESS and M. X. SULLIVAN

(*From the Chemo-Medical Research Institute, Georgetown University, Washington*)

(Received for publication, December 23, 1938)

Van Slyke and Birchard (1) have suggested that the nitrogen liberated when proteins are treated with nitrous acid originates largely from the ε-amino group of lysine. However, the existence of a quantitative relationship between the free amino nitrogen and the lysine content of a protein is believed by Kossel and Gawrilow (2) to be improbable. Edlbacher (3) and Felix (4) observed that certain lysine-free proteins were methylated as readily as those containing lysine. They found, also, that the free amino nitrogen content of histones and of sturine as well as of gelatin and glycinin was considerably greater than could be accounted for by the lysine content of these proteins.

As a result of these observations numerous studies have been made on the effect of nitrous acid upon proteins. The early work has been reviewed by Dunn and Lewis (5). In their own experiments they found that the tyrosine in casein was partially destroyed by deamination but not completely so, as maintained by Skraup (6). By the method of Van Slyke the deaminized casein showed only 54 per cent as much cystine as did the original untreated casein. Wiley and Lewis (7) using the same method found an increase in cystine nitrogen upon deamination. No explanation for this divergence in findings was offered; however, the authors believe the Van Slyke method to be inaccurate for the determination of certain characteristic groups in the protein molecule.

Steudel and Schumann (8) by the use of the Folin-Looney (9) method reported results that indicated a 5-fold increase in the

---

*** A preliminary report of this work was presented before the meeting of the American Society of Biological Chemists at Washington, March, 1936.**

cystine content of casein after it had been treated with nitrous acid. Subsequently Steudel and Wohinz (10), employing the Folin-Marenzi (11) method after the same treatment, obtained a 2-fold increase. No adequate explanation for these large increases was given. White (12) has determined the cystine content of both casein and deaminized casein by means of the copper precipitation method of Vickery and White (13) and judged that the cystine content of the casein was not altered by the action of nitrous acid.

In view of these conflicting findings, it would appear that further work is necessary to establish the effect of nitrous acid upon the cystine content of casein and also of other proteins. Accordingly, casein, lactalbumin, and wool were deaminized by the method of Dunn and Lewis (5). After deamination the respective proteins were tested for free amino nitrogen by the Van Slyke (14) method. Deaminized casein and deaminized lactalbumin, dissolved in 90 per cent formic acid, were negative. The deaminized wool would not dissolve in formic acid but did go into solution in 0.01 N sodium hydroxide with gentle heating for 10 minutes. Undeaminized wool was likewise dissolved in 0.01 N sodium hydroxide. The dilute alkali solutions of both the undeaminized and the deaminized wools were yellow. There may have been some hydrolysis of the protein in both cases. However, the undeaminized wool showed 0.87 per cent amino nitrogen and the deaminized wool 0.15 per cent. The cystine content of these proteins both before and after deamination was determined by the methods of Sullivan (15), Okuda (16), Shinohara (17), Folin-Marenzi (11), and Vickery and White (13).

Table I gives the yield of cystine from the untreated and the deaminized proteins calculated upon an ash- and moisture-free basis. Two types of hydrolysis were employed: 20 per cent HCl for 6 hours, and 8 N H_2SO_4 for 24 hours. The temperature of the bath for both types of hydrolysis ranged between 125° and 130°.

Upon both types of hydrolysis the Sullivan method revealed a marked lowering of the cystine content of the deaminized proteins. For a positive result the Sullivan method requires the presence of the —SH, —NH_2, and —COOH groups in the cysteine molecule to be free (15). With the other methods which require only the presence of a potential —SH group, in general a marked difference between the yield of cystine from the untreated protein and that from the same protein after deamination was not indicated. Both the HCl and the H_2SO_4 hydrolysates gave results of the same order of magnitude. Attention may be called to the fact that the Folin-Marenzi method, in general, gives extraordinary values for the cystine content of the deaminized proteins.

The loss of reactive cystine in the deaminized proteins indicated by the values obtained with the Sullivan method may be due to the deamination of part of the cystine in the protein. The deaminized cystine might be expected to react as did the original cystine when treated according to the methods of Okuda, Shinohara, and Folin-Marenzi, while in the Sullivan method only unchanged cystine would react. The effect of deamination of cystine upon results by the Vickery-White method remained to be determined. Accordingly, cystine itself was deaminized by the method of Neuberg and Ascher (18). The deaminized cystine

TABLE I

Percentage Cystine Content of Untreated and Deaminized Proteins

Method	Hydrolysis	Proteins					
		Casein	Deaminized casein	Lactalbumin*	Deaminised lactalbumin	Wool	Deaminised wool
Sullivan	20% HCl	0.29	0.13	2.23	1.88	13.83	11.04
"	8 N H_2SO_4	0.26	0.11	2.29	1.86	13.49	10.79
Okuda	20% HCl	0.33	0.22	2.50	2.62	13.78	13.06
"	8 N H_2SO_4	0.30	0.24	2.87	2.79	14.06	13.33
Shinohara	20% HCl	0.34	0.24	2.43	2.95	14.01	14.41
"	8 N H_2SO_4	0.45	0.27	2.99	3.09	14.35	15.88
Folin-Marenzi	20% HCl	0.45	1.37	2.62	5.68	15.88	24.55
"	8 N H_2SO_4	0.44	2.04	3.36	3.21		18.66
Vickery-White	20% HCl†	0.27	0.25	2.34	2.38	13.72	13.29
"	8 N H_2SO_4‡	0.29	0.27	2.90	2.70	13.88	13.85

* We have on hand samples of lactalbumin with a considerably higher cystine content.

† Tin was placed in the HCl. The rest of the procedure was that of Vickery and White.

‡ The regular Vickery and White procedure was employed.

contained no nitrogen and, as early shown by Westerman and Rose (19), was absolutely negative in the Sullivan reaction. Analysis of the barium salt of the deaminized cystine, $C_6H_8O_6S_2Ba$, showed 36.02 per cent barium, theory 36.38 per cent; 16.60 per cent sulfur, theory 16.95 per cent. An aliquot of the barium salt, after removal of the barium, was reduced by tin and H_2SO_4. When analyzed by the Vickery and White procedure of precipitation and estimation, 96 per cent recovery was found. It was evident, therefore, that, if deamination of the cystine had occurred, to any extent, in the protein molecule, neither the methods of Okuda, Shinohara, nor of Vickery and White would be effective in showing this loss.

To test this hypothesis 2.0 gm. of deaminized wool were hydrolyzed with 20 cc. of 8 N H_2SO_4 and tin. The hydrolysate was treated according to the procedure of Vickery and White and the volume of the solution obtained after decomposition of the copper mercaptide and subsequent concentration was 50 cc. No cysteine was present in the solution. Aliquots were used in all the subsequent determinations, and the values given are corrected for moisture and ash. The cystine content of the deaminized wool as determined by the Sullivan, Okuda, and Vickery-White methods was 8.94, 12.61, and 13.84 per cent respectively. The total sulfur of the wool, corrected for moisture and ash, was 3.79 per cent, corresponding to 14.21 per cent cystine if no other sulfur compound was present. It may be noted that when the Vickery-White procedure of hydrolysis and precipitation with Cu_2O is used the cystine values are lower than than those given in Table I, especially in the case of the Sullivan method. With the exception of the Vickery-White method the values given in Table I were obtained without the use of tin and without precipitation with Cu_2O. As will presently be shown, the precipitation with Cu_2O involves more or less loss of cystine reactive in the Sullivan method.

In fact it was stated in the original paper of Vickery and White that there was some decomposition of the cysteine during the removal of the copper from the mercaptide. Such was found to be the case when pure cystine was reduced and precipitated with Cu_2O and estimated colorimetrically after decomposition of the mercaptide. Recoveries of 87.3 per cent by the Sullivan method and 94 per cent by the Okuda method were obtained. From the values found for the total nitrogen in the solution of the decomposed mercaptide the recovery of cystine was 89.9 per cent. If these corrections for the loss of cystine are applied to results by the Sullivan and Okuda methods on deaminized wool, the results are 13.41 per cent by the Okuda method and 10.24 per cent by the Sullivan method, as compared with the value of 13.84 per cent calculated from the total sulfur according to the method of Vickery and White. On the other hand, the nitrogen found in the solution of the decomposed mercaptide from the deaminized wool indicated only 10.22 per cent cystine, even after correction for the loss of cystine by the same procedure. This low nitrogen value is, perhaps, indicative that the higher values obtained by the methods of Vickery and White and Okuda are due to the presence of a deaminized cystine.

As shown by Westerman and Rose (19) deaminized cystine is soluble in ether and does not give the cystine or cysteine tests of Sullivan. Cystine, on the other hand, cannot be extracted by ether. Accordingly, an aliquot of the solution of the decomposed mercaptide was extracted several times with ether especially freed from alcohol and peroxides. The combined ether extracts were allowed to evaporate at room temperature and were then made to a definite volume with 0.1 N HCl. This solution gave a positive test for the disulfide linkage with sodium nitroprusside in the presence of NaCN. The Okuda method indicated the presence of 3.07 per cent of a disulfide calculated as cystine. The Sullivan method, however, gave a negative cystine reaction, proving that the substance which gave the positive nitroprusside test and the positive Okuda test was not cystine.

The aqueous residue after ether extraction was tested for cystine by both the Okuda and the Sullivan methods and gave 10.28 and 10.38 per cent respectively. These values are in close agreement with the original value of 10.24 per cent of cystine given by the Sullivan method on the solution of the decomposed mercaptide. The sum of the apparent cystine found by the Okuda method in the ether layer and the water layer is 13.35 per cent, a value close to that found by the same method in the hydrolysate of the deaminized wool.

It is evident from these experiments that there is formed during the deamination of wool a substance with many of the properties of deaminized cystine, which accounts for the higher cystine values given by the Okuda and the Vickery-White procedures than by the Sullivan method.

A similar experiment was performed with deaminized casein with analogous results. The total nitrogen in the solution of the decomposed mercaptide was insufficient to account for the amount of cystine indicated by the methods of Okuda and Vickery and White. The ether extract of the solution of the decomposed mercaptide gave a positive test for the disulfide linkage. The experiments with deaminized casein similar to those with deaminized wool showed that the nitrogen of the cystine had been altered by the action of nitrous acid.

An interesting finding is that the large increase in the cystine value of the deaminized casein indicated by the Folin-Marenzi procedure upon the original hydrolysate disappeared when the method was applied to the solution of the decomposed mercaptide and a value closely approximating those given by the Vickery-White and the Okuda methods was obtained. Evidently this is another case in which, in the original hydrolysate, the Folin-Marenzi reagent reacts with some reducing substances, unrelated to cystine, or even deaminized cystine.

SUMMARY

The cystine content of deaminized proteins as determined by the Sullivan method is markedly lower than that of the corresponding untreated proteins. The other methods, except that of Folin and Marenzi, which gave very high values for the deaminized proteins, indicated that the cystine content of the deaminized proteins was much the same as that of the untreated proteins.

Evidence is given that by the action of nitrous acid on proteins the cystine combined in the protein molecule is altered. This alteration involves the amino group and in so far as this happens the value by the Sullivan method is lowered.

BIBLIOGRAPHY

1. Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, **16**, 539 (1913-14).
2. Kossel, A., and Gawrilow, N., *Z. physiol. Chem.*, **81**, 274 (1912).

3. Edlbacher, S., *Z. physiol. Chem.*, **107**, 52 (1919); **108**, 287 (1919); **110**, 153 (1920).
4. Felix, K., *Z. physiol. Chem.*, **110**, 217 (1920).
5. Dunn, M. S., and Lewis, H. B., *J. Biol. Chem.*, **49**, 327 (1921).
6. Skraup, Z. H., *Monatsh. Chem.*, **27**, 653 (1906); **28**, 447 (1907).
7. Wiley, F. H., and Lewis, H. B., *J. Biol. Chem.*, **86**, 511 (1930).
8. Steudel, H., and Schumann, R., *Z. physiol. Chem.*, **183**, 168 (1929).
9. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
10. Steudel, H., and Wohinz, R., *Z. physiol. Chem.*, **196**, 78 (1931).
11. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
12. White, A., *J. Biol. Chem.*, **103**, 295 (1933).
13. Vickery, H. B., and White, A., *J. Biol. Chem.*, **99**, 701 (1932–33).
14. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).
15. Sullivan, M. X., *Pub. Health Rep.*, *U. S. P. H. S.*, suppl. 78 (1929).
16. Okuda, Y., *J. Biochem.*, Japan, **5**, 217 (1925).
17. Shinohara, K., *J. Biol. Chem.*, **109**, 665 (1935).
18. Neuberg, C., and Ascher, E., *Biochem. Z.*, **5**, 451 (1907).
19. Westerman, B. D., and Rose, W. C., *J. Biol. Chem.*, **79**, 413 (1928).

ON THE SITE OF THE FORMATION OF CITRIC ACID IN THE ANIMAL ORGANISM*

BY JAMES M. ORTEN† AND ARTHUR H. SMITH

(*From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*)

(Received for publication, December 27, 1938)

Citric acid is a common constituent of many animal tissues and body fluids and is now considered to be a normal metabolite. Some of the factors governing the endogenous formation of this compound and the identity of certain substances which appear to serve as its precursors are now known (8, 4). However, the locus of the formation of citric acid in the animal organism is still to be determined.

Several years ago it was observed (9) that, after citric acid excretion was increased in the rabbit by the administration of sodium bicarbonate, the citric acid content of blood taken from the renal vein was slightly greater than that of heart blood. This finding was interpreted as evidence that the kidney forms citric acid. However, the results of a later similar study (5) do not substantiate this finding. Blood from the renal vein was found to contain no more, or perhaps even slightly less, citric acid than heart blood. Furthermore, failure to form significant amounts of citric acid from certain substances by kidney tissue has been observed in a few *in vitro* studies[1,2] (3). Recent work on the dog (12), on the other hand, might be interpreted as supporting the view that the kidney forms citric acid. In these studies, it was observed that after the intravenous administration of sodium malate a large amount of citrate was excreted in the urine, whereas only a small increase in the citric acid content of the blood occurred. Of course, the possibility that citric acid formed in other tissues was merely rapidly "cleared" from the blood stream by the kidney, rather than formed therein, is not precluded.

---

* A preliminary report was made before the American Society of Biological Chemists at Memphis, April, 1937.

† Charles Pfizer and Company Fellow, Yale University, 1936-37.

[1] However, mention should also be made of a recent paper (2) (not available in the original) which describes *in vitro* studies with testes, kidney, liver, and muscle. When malate was used in the reaction medium, kidney tissue formed the largest amount of citric acid. Testicular tissue also formed a significant though somewhat smaller quantity.

[2] Orten, J. M., and Smith, A. H., unpublished observations.

No detailed study of citric acid formation in other tissues has been made. There is some *in vitro* evidence that the testes may form citrate from malate (3). The liver has received some consideration as a possible site of citric acid formation. However, the facts that this tissue has been found to contain less citric acid than other tissues (10) and that following hepatectomy there appears to be an increase rather than a decrease in citric acid excretion (1) lend little support to the possibility that the liver forms citric acid. Indeed, it has been suggested (1) that the liver is an important site of the destruction of citric acid. *In vitro* studies, in which liver slices or extracts are used, appear to support this suggestion (6, 7, 11). Citric acid destruction may also occur in muscle, inasmuch as this tissue contains an active citric acid dehydrogenase (see (11)).

The present investigation was designed to study further the problem of the site of citric acid formation.

EXPERIMENTAL

The procedure chosen for studying the site of citric acid production was that of comparing the citrate content of various organs and tissues after the stimulation of citric acid formation. Inasmuch as the parenteral administration of sodium malate to dogs greatly increases the output of citric acid in the urine (8), this device was adopted as the means of augmenting citric acid production. Since it seemed impractical to use dogs for the proposed study, the rat was used in this investigation.

It was necessary first to determine whether the rat, like the dog, excretes increased amounts of citric acid in the urine after the parenteral administration of sodium malate. For this purpose, normal adult male rats were placed in metabolism cages and were fed a constant amount of a citrate-low basal diet for a 5 day preliminary period. This diet had the following composition: casein 18 per cent, dextrin 51 per cent, hydrogenated cottonseed oil 27 per cent, and salt mixture (Wesson (13)) 4 per cent. The rats were then lightly anesthetized with ether and a sterile solution of either sodium chloride (2.5 per cent) or sodium malate (5 per cent) was injected into the heart in amounts supplying 100 mg. of sodium per kilo of body weight. The urine was collected quantitatively during 24 hour periods both before and after the injection of the chloride or malate, and the citric acid content of aliquots of the samples was then determined by a photometric method (10).

The results obtained on groups of six rats each showed that the rat, like the dog, excretes increased amounts of citric acid in the urine after the parenteral administration of sodium malate. The average daily excretion of the chloride-injected controls was 1.9 mg., whereas that of the malate-treated animals was 9.4 mg.

It was also necessary to determine whether rapid excretion of extra citrate occurs in the rat, as it does in the dog (12), after malate injection. For this purpose, two groups of rats were injected with chloride or malate, as described; in these animals, however, the discharge of urine from the bladder was prevented by closure of the external urethral orifice with a ligature. 30 minutes after the injection was made, the animal was again anesthetized, a mid-line incision was made, and the urinary bladder was isolated. The ureters and urethra were closed by a ligature and the bladder with its contained urine was carefully removed. The urine was collected quantitatively and the citric acid content of aliquot samples was then determined.

The data obtained on groups of six animals each showed that the rat, like the dog, promptly excretes increased amounts of citric acid in the urine after the injection of sodium malate. The average amount of citrate excreted in 30 minutes by the chloride-injected controls was 0.19 mg., whereas that of the malate-treated animals was 1.54 mg. Furthermore, it appears that if citric acid formation, after malate injection, is localized in any one organ or tissue of the rat sufficient amounts should be formed within 30 minutes to permit the detection of the newly formed compound in the tissue in which it is produced. It seems probable that after a longer period diffusion of the citric acid into the blood stream and perhaps into other tissues might obscure its possible origin at a particular site.

The foregoing experiments demonstrated that the rat is suitable for the proposed studies on the site of citric acid formation. Therefore, animals were injected with either chloride or malate as in the preliminary experiments, but at intervals of 15 or 30 minutes they were exsanguinated by being bled from the abdominal aorta. The tissues to be studied were then quickly removed and frozen with solid carbon dioxide. They were finely pulverized in a cold steel mortar, weighed, transferred quantitatively to a volumetric flask, and extracted with dilute sulfuric acid for 1 to 2 hours. The protein was precipitated by the addition of trichloroacetic acid and the citric acid content of aliquots of the protein-free filtrate was determined. The blood citric acid was estimated on trichloroacetic acid filtrates.

DISCUSSION

The results obtained on the animals of the various groups, each composed of five to eight rats, are summarized in Table I. It is evident from these data that there was little difference in the citric acid content of the blood, liver, and muscle of the control and the malate-injected rats, either 15 or 30 minutes after the injection. However, it is of interest that the livers of all animals contained less citrate than did the muscles or the blood. This finding may be interpreted as further evidence that citric acid is destroyed in the liver (1). The kidneys, on the other hand, consistently contained more citric acid than did any of the other tissues studied. Moreover, the kidneys of the groups given malate, in each instance contained more than twice as much citric acid as was found in those of the chloride-injected controls. Each of these findings may be interpreted as evidence that the kidney is an important site of citric acid formation in both normal and malate-treated rats.

The possibility exists, however, that the "extra" citric acid present in the kidneys of the above animals may have been formed in other tissues and that this surplus merely represents a renal accumulation of citrate which is being cleared from the blood by the kidneys. If this were the case, then it follows that there should be a detectable increase in the citric acid content of the blood, or perhaps of other tissues, of rats given malate, but in which the excretion of citrate by the kidney has been prevented.

Table II shows a summary of the data obtained on ten rats bilaterally nephrectomized immediately preceding the injection. As in the other experiments, intervals of 15 or 30 minutes were allowed to elapse between the time of the injection and the exsanguination and removal of the tissues.

TABLE I

Citric Acid Content of Tissues of Control Rats and Rats Injected with Malate

Interval after injection	Tissue	Citric acid content, mg. per cent			
		Chloride-injected		Malate-injected	
		Average	Range	Average	Range
min.					
15	Blood	4.0	3.3–4.8	4.0	3.4– 4.3
	Liver	1.6	1.0–2.2	1.4	1.2– 1.7
	Muscle	2.7	1.7–3.7	2.4	1.5– 2.9
	Kidney	6.1	3.0–7.0	12.6	8.0–16.1
30	Blood	3.2	2.9–3.6	4.4	3.4– 5.6
	Liver	2.6	1.7–4.6	2.7	1.9– 3.6
	Muscle	3.1	2.6–3.4	3.4	2.5– 4.7
	Kidney	6.3	5.5–7.0	14.9	11.6–17.0

TABLE II

Citric Acid Content of Tissues of Control Rats and Nephrectomized Rats Injected with Malate

Interval after injection	Tissue	Citric acid content, mg. per cent			
		Chloride-injected		Malate-injected	
		Average	Range	Average	Range
min.					
15	Blood	4.6	2.9–7.5	4.7	2.5–6.3
	Liver	2.5	1.7–3.1	2.3	1.4–2.7
	Muscle	4.2	2.9–5.2	3.3	2.2–4.2
30	Blood	7.5	6.7–7.8	7.7	7.2–8.2
	Liver	3.1	2.3–3.8	3.3	2.7–4.0
	Muscle	5.2	4.4–6.7	5.6	3.9–6.5

These data show that there is no significant difference between the citric acid content of the blood, liver, and muscle of nephrectomized rats receiving malate and that of those receiving chloride, either 15 or 30 minutes after the injection. It is evident that the increased amount of citric acid present in the kidneys of the intact, malate-injected rats of the preceding experiment cannot be accounted for in any tissues of the nephrectomized animals examined. A slight difference between the average values obtained in the animals whose tissues were removed 30 minutes after injection and those studied at the end of a 15 minute interval was found, the citric acid content of the former being slightly greater than that of the latter. An explanation of this finding cannot be given at the present; however, such factors as prolonged hyperpnea during the operation, a possible effect of ether anesthesia, or of tissue damage may be involved. These data suggest that there *may* be an extrarenal source of citric acid. This citric acid, however, is not derived from injected malate, inasmuch as a comparable amount was found in the tissues of the chloride-treated controls. It appears, therefore, that the citric acid formed after the injection of sodium malate into rats, under the conditions employed, either is produced chiefly in the kidney or is dependent upon the presence of the kidney for its formation.

SUMMARY

The rat, like the dog, rapidly excretes increased amounts of citric acid in the urine after the parenteral administration of sodium malate.

Determinations of the citric acid content first of liver, blood, muscle, and kidney of intact rats and then of the liver, blood, and muscle of bilaterally nephrectomized animals lead to the conclusion that, under the experimental conditions employed, the citric acid formed following the injection of sodium malate into rats either is produced chiefly in the kidney or is dependent upon the presence of the kidney for its formation.

BIBLIOGRAPHY

1. Boothby, W. M., and Adams, M., *Am. J. Physiol.*, **107**, 471 (1934).
2. Hallman, N., and Simola, E., *Suomen Kemistilehti*, **11B**, 21 (1938); *Chem. Abst.*, **32**, 9217 (1938).
3. Krebs, H. A., and Johnson, W. A., *Enzymologia*, **4**, 148 (1937).
4. Krebs, H. A., Salvin, E., and Johnson, W. A., *Biochem. J.*, **32**, 113 (1938).
5. Kuyper, A. C., and Mattill, H. A., *J. Biol. Chem.*, **103**, 51 (1933).
6. Langecker, H., *Biochem. Z.*, **273**, 43 (1934).

7. Martius, C., and Knoop, F., *Z. physiol. Chem.*, **246**, 1 (1937).
8. Orten, J. M., and Smith, A. H., *J. Biol. Chem.*, **117**, 555 (1937).
9. Östberg, O., *Skand. Arch. Physiol.*, **62**, 81 (1931).
10. Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, **113**, 235 (1936).
11. Reichel, L., and Neeff, A., *Z. physiol. Chem.*, **240**, 163 (1936).
12. Smith, A. H., and Orten, J. M., *J. Biol. Chem.*, **124**, 43 (1938).
13. Wesson, L. G., *Science*, **75**, 339 (1932).

INFLUENCE OF OXYGEN AND CARBON DIOXIDE ON THE BLOOD OF NORMAL AND PNEUMONIC DOGS*

By DAVID J. COHN, ALBERT TANNENBAUM, WILLIAM THALHIMER, and A. BAIRD HASTINGS

(From the Biochemical Laboratories of the Michael Reese Hospital and the Lasker Foundation for Medical Research of the University of Chicago, Chicago)

(Received for publication, January 11, 1939)

For almost two decades, the administration of oxygen-enriched air has been widely used to alleviate the anoxemia, brought about by interference with gas exchange in the lungs, that may accompany pneumonia. Oxygen administration in pneumonia is based mainly on the work of Haldane (1), Barcroft *et al.* (2), Stadie (3), Meakins (4), Davies and Gilchrist (5), Binger (6), Barach (7), and Boothby and Haines (8). From their work and that of others, certain facts have been found regarding the functional pathology of pneumonia. First, if anoxemia occurs, it may cause untoward symptoms, such as weakness and malaise, along with a characteristically rapid and shallow respiration (9) that may increase the already existing anoxemia (10). Second, the proper administration of oxygen usually increases the oxygen content of the blood and relieves the cyanosis and untoward symptoms, thus conserving the patient's strength. Thus, oxygen therapy appears to relieve the anoxemia accompanying pneumonia. Though it is difficult to obtain direct evidence to show that administration of oxygen shortens the course of the disease or hastens the occurrence of the crisis, yet both theory and experience point to the value of early and continuous application of this gas.

The first paper on the addition of carbon dioxide to the inspired gas in pneumonia was published by Henderson, Haggard, Coryllos, Birnbaum, and Radloff (11) in 1930. In this paper, the authors

---

* Aided by a grant from the Linde Air Products Company.

list certain factors peculiar to pneumonia that suggested the use of carbon dioxide. One factor is "occlusion of an infected organ and lack of drainage." Another factor given is that, "In general, depressed or shallow breathing tends to permit the development of pneumonia, and deep breathing with full ventilation of the lungs tends strongly to inhibit it." The experimental work was performed on dogs. Obstructive atelectasis was produced in some animals and pneumococcal atelectasis, by the insufflation of virulent cultures directly into the bronchi, in others. These dogs were placed in a closed chamber containing 93 per cent oxygen and 7 per cent carbon dioxide for periods of from 30 minutes to 24 hours. The beneficial effects of the treatment were indicated by x-ray examinations. In many cases, the pneumonia cleared up rapidly.

Though the above mentioned paper was the first to deal with the use of carbon dioxide in pneumonia, the rôle of carbon dioxide has been previously considered in many pathological conditions. In 1908, Henderson (12) suggested that acapnia, or deficiency of carbon dioxide in the blood and tissues, is an important factor contributing to the depression of respiratory and circulatory activity which accompanies operation and anesthesia. Henderson and Haggard (13) in 1920 demonstrated that carbon dioxide may be used to stimulate the respiratory center. In 1922, they reported the use of oxygen and carbon dioxide in carbon monoxide poisoning (14). In 1929, Coryllos and Birnbaum (15), on the basis of bronchoscopic investigations on dogs, considered lobar pneumonia to be a "pneumococcal atelectasis," caused by an obstruction of a bronchus by the production of an excessive and sticky secretion. At about the same time, Henderson and his collaborators (16–18) advocated the use of carbon dioxide in the prevention of pneumonia. This was followed by the paper concerned with carbon dioxide therapy in pneumonia (11) and has since been more fully discussed (19).

The present studies were undertaken with the following questions in mind. First, what are the changes in oxygenation and acid-base balance in pneumonia? Second, what are the effects of the breathing of air containing high carbon dioxide percentages on the oxygen saturation and the acid-base balance of the blood of pneumonic dogs?

From a consideration of facts derived from these experiments, along with certain theoretical and experimental clinical studies in the literature, one might be able to obtain further information of the possible effect, in pneumonia, of the application of carbon dioxide, and carbon dioxide with oxygen.

Outline of Experiments

In order to have subjects in which the pneumonic effects were uncomplicated by other pathological conditions or by medication, and in which numerous arterial blood samples could be conveniently taken, it was decided to carry out the studies upon experimental animals. An experimental pneumonia in dogs was produced which brought about an anoxemia comparable to that occurring in human patients. The pathological findings and symptoms were also similar.

The investigation consisted of five series of experiments: (1) determination of the normal physiological variations in oxygenation and acid-base balance occurring in the blood of dogs. The results obtained in this group of experiments formed the basis with which the results of the experiments in the subsequent groups were compared; (2) determination of the effect of experimental pneumonia on the oxygenation and acid-base balance of the blood; (3) determination of the effect of the breathing of air enriched with oxygen alone on normal dogs and on anoxemic dogs; (4) determination of the effect of the breathing of air to which carbon dioxide had been added, on normal and pneumonic dogs; (5) determination of the effect of the breathing of air enriched with both oxygen and carbon dioxide, on normal and pneumonic dogs.

Methods and Apparatus

Selection, Care, and Training of Dogs—The dogs used for these experiments were carefully picked for their health, weight, and gentleness. Only dogs weighing between 20 and 30 pounds were used. They were kept in good surroundings with access to an open-air enclosure and were fed a diet consisting of meat, bread, and vegetables.

In order to eliminate physiological changes due only to extraneous factors such as excitement and blood sampling, the dogs were fully familiarized in practice periods with all experimental procedures which they were to undergo. At the beginning of each experiment, the dogs were carried from their kennels to the site of the experiment so as to eliminate any effect of physical exertion.

Production of Pneumonia—Since one of the objects was to produce as severe an anoxemia as possible, compatible with life, it seemed that a pneumonia produced by chemical irritation with its accompanying marked edema would be satisfactory for our purposes. It was found that the intrabronchial injection (under ether anesthesia) of about 1 cc. of 1 per cent hydrochloric acid per kilo of body weight produced an experimental pneumonia in 24 to 48 hours. The respirations were increased in depth and rate, averaging 61 per minute in comparison with the normal of 14 to 20 per minute. Both gross and microscopic examination of the lungs revealed that the pneumonia produced was of the lobular type and was in every respect comparable to a spontaneous bronchopneumonia in humans except that there was more hemorrhage, edema, and necrotizing bronchitis than is usually seen in spontaneous cases. It may be mentioned that the amount of acid injected is insufficient to produce by itself significant acid-base balance changes in the body as a whole, particularly changes which would last for 24 hours. This can be shown by theoretical calculations or from a study of the results in Series 2 (Table III).

Cage—A special cage, as shown in Fig. 1, was constructed for the experiments. Upon the metal base (*B*) rested an air-tight, glass-walled cage (*A*), which could be raised or lowered by *E* to admit or remove the animal. It measured 3 × 3 × 2 feet. The metal tubes (as *G*) were for the introduction of the various gases and the sampling of the atmosphere within. A soda lime container (*H*), to which was attached a circulating blower, was used for the continuous removal of carbon dioxide when desired, without admixture of any air from the outside. A coil (*I*), through which cold water could be circulated for the purpose of cooling and removing water vapor, was built into the rear of the cage. In the anterior wall, there was a circular opening (*F*) provided with an air-tight gasket and a sleeve of flexible rubberized cloth. Through this sleeve, the hind quarters of the dog could be drawn. The sleeve closely encircled the dog's body, so that the atmosphere in the cage was not disturbed by the procedure. Blood samples could thus be obtained from the femoral artery, while the dog continued to breathe the atmosphere in the cage. A basin of drinking water was left in the cage and a false bottom (*D*) protected the dog from its excreta. Urine was collected in a water-sealed basin (*J*).

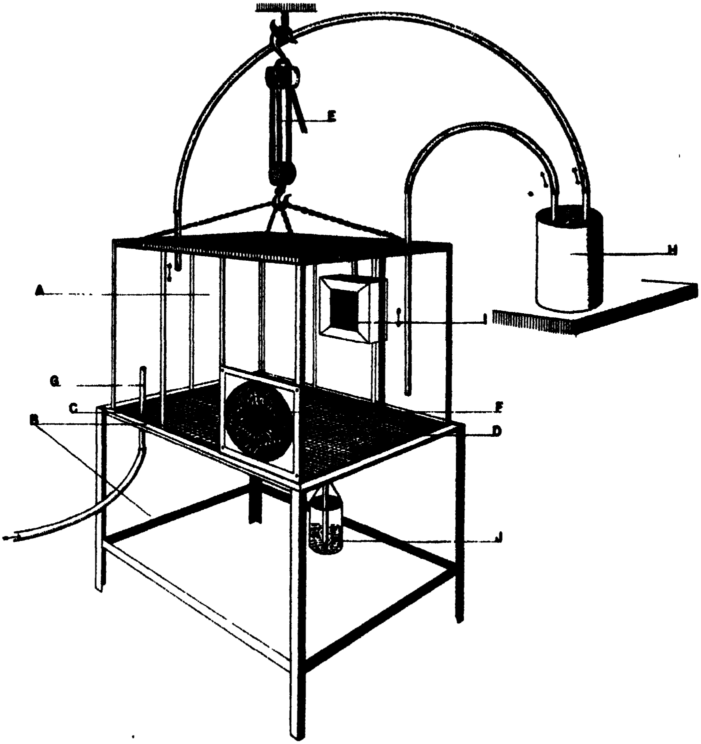


Fig. 1. *A* represents an air-tight metal and glass cage, without bottom; *B*, specially constructed stand, upon which the cage rests, and which is the floor for *A*; *C*, rubber gasket providing an air-tight fit between *A* and *B*. The weight of the cage upon this gasket is sufficient to insure air-tightness; *D*, sloping sheet metal drainage floor, covered by a horizontal wire mesh; *E*, tackle for raising the cage; *F*, an opening, provided with a sleeve of rubberized material through which the animal's hind legs may be drawn, while an air-tight closure is maintained around its body; *G*, entrance tube for experimental gases; *H*, apparatus for recirculation of the atmosphere in the cage through soda lime, when it is desired to remove carbon dioxide; *I*, air-conditioning apparatus; *J*, water trap for equalizing pressures and eliminating urine.

Collection and Analysis of Blood Samples—The experiments were begun at least 12 hours after the animals had been last fed. Samples of blood were collected under oil from the femoral artery. One-half of each specimen was collected without anticoagulant; to the other half sufficient neutral potassium oxalate was added to prevent coagulation. Transfers were made under oil at all times. The contents of both tubes were gently stirred, in the first to assist defibrination and clotting, and in the second to avoid clotting. The sample of defibrinated blood was centrifuged and the serum analyzed for carbon dioxide content and pH. The sample of oxalated blood was analyzed for oxygen content and oxygen capacity. The oxygen content, oxygen capacity, and carbon dioxide content were determined by the methods of Van Slyke and Neill (20). The Hastings and Sendroy (21) method was used for the pH determinations. From the colorimetric pH values as read at 38°, 0.10 was subtracted in order to correct them to the electrometric values for dog blood at that temperature. All analyses were performed in duplicate.

Calculations—From the pH and the carbon dioxide content of the serum, the carbon dioxide tension of the blood was calculated in the usual manner with the following formulæ derived from the Henderson-Hasselbalch equations.

$$\text{pH}_s = 6.10 + \log \frac{(\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s}{(\text{H}_2\text{CO}_3)_s} \tag{1}$$

$$p\text{CO}_2 = \frac{(\text{H}_2\text{CO}_3)_s}{0.0301} \tag{2}$$

$$(\text{HCO}_3)_s = (\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s \tag{3}$$

In order to determine whether there had been a net change in the bicarbonate concentration of specimens drawn after the first sample, the results were corrected by calculation to that which they would have been had there been no change in pH between the drawing of the first and subsequent samples. The figure so obtained, designated as $(\text{HCO}_3)_1$, was calculated from the formula $(\text{HCO}_3)_1 = (\text{HCO}_3)_2 + 23(\text{pH}_2 - \text{pH}_1)$ in which pH_1 = the serum pH of the first blood sample, pH_2 = the serum pH of a subsequent blood sample, $(\text{HCO}_3)_2$ = the bicarbonate concentration of the serum of the subsequent blood sample, 23 = a constant which is the average buffer value of the "true" serum of dog blood, determined by experiment, $(HCO_3)_1$ = the serum bicarbonate concentration corrected to the initial pH. This value is used as the basis for subsequent comparisons and discussion.

The significant data on the degree of oxygenation and the acid-base balance are presented in condensed form in Tables I to IV. Complete data of representative experiments are given in Protocols 1 and 2.

EXPERIMENTAL

Series 1. Normal Physiological Variations in Oxygenation and Acid-Base Balance of Blood—The spontaneous changes in the blood factors studied in the absence of any experimental procedure were first determined. To this end, a group of seventeen, healthy, normal dogs, breathing ordinary air, and not in the experimental cage, was bled on two successive mornings and the samples of blood were analyzed as described. From Table I, in which the results are listed, it will be seen that the oxygen saturation in the group varied from 90 per cent to 98 per cent. The variations from day to day in individual animals were small, never beyond 3 per cent. Variations in the acid-base balance, as indicated by carbon dioxide tension and bicarbonate content, also were slight. The largest change in pH from day to day in any one animal was 0.07, with an average of −0.01 (normal range 7.30 to 7.44). The largest change in the carbon dioxide tension, pCO_2, was 7.9 mm. of Hg, with an average of −0.5 (normal range 27.4 to 39.8 mm. of Hg). The bicarbonate content, $(HCO_3)_1$, of the blood plasma showed a maximum difference of 4.4 mM per liter, with an average of −0.61 (normal range 15.5 to 23.8 mM per liter).

In addition to the above animals, another group of eight normal, healthy dogs was also kept in ordinary air, but in the experimental cage. At approximately hourly intervals for 6 hours, arterial blood samples and simultaneous samples of the gas in the cage were withdrawn and examined. By these experiments, the normal variations that could occur in the length of time usually taken in each of the subsequent experiments were determined. This second normal group brought to light any changes which might be due to the mechanical procedure to which the animals were subjected, or to the repeated bleedings. The air in the cage was continuously renewed and remained constant at 21 per cent oxygen and less than 0.1 per cent carbon dioxide. The results of the experiments in this series are shown in Table II. The average change in oxygen saturation of the blood was −0.4 per cent; in pH, 0; in carbon dioxide tension, +1.5 mm. of mercury; and in serum bicarbonate, +0.8 mM per liter.

TABLE I

Day to Day Variation in Oxygen Saturation and Acid-Base Balance of Blood of Normal Dogs

Experiment No.	O_2 saturation		pH_s			CO_2 tension			Serum $(HCO_3)_1$*		
	1st day	2nd day	1st day	2nd day	Difference	1st day	2nd day	Difference	1st day	2nd day	Difference
	per cent	*per cent*				*mm. Hg*	*mm. Hg*	*mm. Hg*	*mM per l.*	*mM per l.*	*mM per l.*
1	90	91	7.40	7.41	+0.01	39.8	38.2	−1.6	23.8	23.8	0
2	96	96	7.34	7.37	+0.03	34.6	31.2	−3.4	18.1	18.3	+0.2
3	96	95	7.39	7.40	+0.01	35.2	33.6	−1.6	20.8	20.3	−0.5
4	96	93	7.41	7.41	0	29.2	28.2	−1.0	18.3	17.3	−1.0
5	93	95	7.41	7.40	−0.01	30.3	30.3	0	18.8	18.1	−0.7
6	95	96	7.38	7.35	−0.03	33.3	32.0	−1.3	19.0	16.5	−2.5
7	95	93	7.44	7.40	−0.04	30.1	27.4	−2.7	19.9	15.5	−4.4
8	96	97	7.42	7.35	−0.07	28.7	36.6	+7.9	18.1	18.0	−0.1
9	95	95	7.35	7.35	0	30.8	32.2	+1.4	16.5	17.2	+0.7
10	92	92	7.37	7.36	−0.01	35.6	37.5	+1.9	19.9	20.3	+0.4
11	92	91	7.39	7.38	−0.01	37.8	37.5	−0.3	22.3	21.9	−0.4
12	95	97	7.35	7.35	0	36.6	33.0	−3.6	19.5	17.7	−1.8
13			7.38	7.35	−0.03	33.9	32.8	−1.1	19.2	18.8	−0.4
14	92	92	7.41	7.40	−0.01	35.8	37.8	+2.0	22.1	22.5	+0.4
15	91	92	7.33	7.37	+0.04	35.9	30.9	−5.0	18.3	18.1	−0.2
16	98	96	7.34	7.35	+0.01	38.2	36.2	−2.0	19.9	19.6	−0.3
17	95	93	7.30	7.30	0	35.2	35.6	+0.4	16.7	16.9	+0.2
18†	92	92	7.34	7.34	0	33.9	35.2	+1.2	17.7	18.4	+0.7
19†	97	96	7.36	7.33	−0.03	37.9	38.2	+0.3	20.7	18.7	−2.0
Average...	94	94			−0.01			−0.5			−0.61

* $(HCO_3)_1$ = serum (HCO_3) of the 2nd day calculated to the (HCO_3) value at the pH of the 1st day. See the section on calculations. This also applies to Tables II to IV and Protocols 1 and 2.

† The same dogs as were used in Experiments 10 and 14, but about 2 weeks later, were used for Experiments 18 and 19 respectively.

It was concluded, therefore, that the spontaneous changes in

TABLE II

Hourly Variation in Oxygen Saturation and Acid-Base Balance of Blood of Normal Dogs

Experiment No.	O_2 saturation				pH_s				CO_2 tension				Serum $(HCO_3)_s$			
	Maximum	Minimum	Average	Average deviation from initial	Maximum	Minimum	Average	Average deviation from initial	Maximum	Minimum	Average	Average deviation from initial	Maximum	Minimum	Average	Average deviation from initial
	per cent	*per cent*	*per cent*	*per cent*					*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
101	94	92	93	0	7.40	7.35	7.37	0	41.5	38.2	39.4	+0.5	23.7	21.0	22.0	+0.3
102	94	93	93	0	7.40	7.32	7.36	−0.02	39.9	30.6	33.7	+0.1	20.2	14.8	18.2	−1.0
103	95	91	93	−2	7.43	7.32	7.39	+0.07	36.9	29.9	33.3	+3.4	23.9	15.0	21.3	+6.3
104	99	95	97	−3	7.48	7.44	7.46	−0.02	28.2	26.9	27.5	+0.6	19.4	17.4	18.2	−1.2
105	93	91	93	0	7.33	7.31	7.32	0	35.9	25.9	30.7	−1.2	18.5	13.6	15.5	−0.5
106	95	92	93	+1	7.45	7.33	7.39	−0.06	36.6	24.3	31.3	+7.0	18.4	14.1	16.7	+0.3
107	95	93	94	0	7.32	7.29	7.31	+0.02	29.6	25.9	27.6	+1.4	15.5	12.2	14.3	+2.1
108	93	90	91	+1	7.35	7.34	7.35	0	34.9	32.2	33.2	+0.6	18.7	17.3	17.7	+0.3
Average..				−0.4				0				+1.5				+0.8

oxygenation and acid-base balance of the arterial blood of normal dogs were small and of the same magnitude, both at hourly and day to day intervals.

Series 2. Effect of Pneumonia upon Oxygenation and Acid-Base Balance of Blood—The percentage oxygen saturation and acid-base balance of the blood of eighteen healthy dogs were first determined. Pneumonia was then produced by the introduction of HCl into the lungs, as described under "Methods." This caused severe anoxemia, such that the percentage oxygen saturation of the arterial blood fell within 24 hours from the original average level of 95 per cent to an average of 77 per cent; in an extreme instance the oxygen saturation fell to 58 per cent. The changes in the acid-base balance (Table III), however, were no greater than those occurring in normal dogs. The largest change in pH was 0.05 with an average of 0. The maximum change in carbon dioxide tension, $p\mathrm{CO}_2$, was 6.7 mm. of Hg, with an average of −1.6, while the largest variation in the bicarbonate content, $(\mathrm{HCO}_3)_1$, was 3.7 mM per liter, with an average of −0.6. It therefore appears that the pneumonia in these dogs was accompanied by no significant immediate effect upon the acid-base balance of the blood.

For subsequent comparisons with pneumonic dogs that were to be subjected to experimental gases over the same time periods, another group of pneumonic dogs that were breathing ordinary air, not in the experimental cage, was bled at hourly intervals for 6 hours. These dogs showed an average hourly difference in oxygen saturation of +1 per cent, in pH of +0.01, in carbon dioxide tension of +0.7 mm. of Hg, and in serum bicarbonate of +0.9 mM per liter (Table IV, D). Although the hourly variations in oxygenation of the blood were slightly greater than in the normal animals, probably depending on the course of the pneumonia, the acid-base changes were again insignificant.

Comparison of Tables I, II, III, and IV, D reveals the fact that the acid-base differences obtained in pneumonic dogs fell within the range obtained for normal dogs. These results are similar to those observed in pneumonia patients by Hastings *et al.* (22) and Binger *et al.* (23).

Series 3. Effect of Oxygen on Normal and Pneumonic Dogs—Only negligible changes had occurred in both the oxygen saturation and the acid-base balance of the blood of normal animals over hourly intervals or from day to day. In animals with pneumonia, oxygen saturation had been reduced, but the acid-base changes had remained of the same order as in normal animals.

TABLE III

Effect of Experimental Pneumonia on Oxygen Saturation and Acid-Base Balance of Blood

Experiment No.	Respiratory rate in pneumonia*	O_2 saturation			pH_s			CO_2 tension			Serum $(HCO_3)_s$		
		Normal	Pneumonia	Difference	Normal	Pneumonia	Difference	Normal	Pneumonia	Difference	Normal	Pneumonia	Difference
		per cent	*per cent*	*per cent*				*mm. Hg*	*mm. Hg*	*mm. Hg*	*mM per l.*	*mM per l.*	*mM per l.*
128	74	94	82	−12	7.33	7.34	+0.01	39.4	35.8	−3.6	20.2	19.0	−1.2
129	62	95	76	−19	7.34	7.39	+0.05	36.7	34.3	−2.4	19.2	21.2	+2.0
130	42	96	84	−12	7.38	7.37	−0.01	44.0	43.2	−0.8	25.3	24.0	−1.3
131	63	99	77	−22	7.35	7.36	+0.01	38.6	39.4	+0.8	20.7	21.8	+1.1
132	35	93	83	−10	7.36	7.32	−0.04	38.8	45.5	+6.7	21.2	21.8	+0.6
133	63	96	73	−23	7.36	7.37	+0.01	41.6	35.7	−5.9	22.8	24.2	+2.6
134		97	85	−12	7.33	7.36	+0.03	31.4	28.7	−2.7	16.0	16.4	+0.4
135	47	94	71	−23	7.36	7.41	+0.05	35.5	32.7	−2.8	19.5	21.3	+1.8
136	36	96	76	−20	7.35	7.33	−0.02	34.7	30.1	−4.6	18.5	14.9	−3.6
137	64	92	77	−15	7.37	7.36	−0.01	35.6	30.2	−5.4	20.0	16.3	−3.7
138	84	94	58	−36	7.34	7.30	−0.04	32.8	34.6	+1.8	17.2	15.6	−1.6
139	63	98	67	−31	7.34	7.32	−0.02	33.6	35.1	+1.5	17.6	17.1	−0.5
140	84	93	73	−20	7.32	7.31	−0.01	36.3	32.1	−4.2	18.1	15.4	−2.7
141	74	97	80	−17	7.37	7.35	−0.02	32.2	31.6	−0.6	17.9	16.4	−1.5
142	40	98	81	−17	7.35	7.33	−0.02	37.3	34.5	−2.8	19.9	17.1	−2.8
143	62	94	78	−16	7.33	7.35	+0.02	36.5	33.9	−2.6	18.9	18.6	−0.3
145		96	82	−14	7.37	7.37	0.00	35.3	32.7	−2.6	19.8	18.3	−1.5
146	90	90	76	−14	7.37	7.38	+0.01	30.4	31.8	+1.4	17.1	18.5	+1.4
Average	61	95	77	−18			0			−1.6			−0.6

* The respiratory rates of the healthy dogs before the production of pneumonia were not taken in all cases, but varied from about 14 to 20 per minute.

With this information concerning normal and pneumonic animals at hand, the effect of inhalation of air high in oxygen was next considered.

Normal dogs were placed in the experimental chamber of con-

TABLE IV—*Oxygen Saturation and Ac*

Dogs	Experimental conditions				O_2 saturation					pH_s		
	Atmosphere	Experiment No.	Analysis of cage atmosphere		Normal before pneumonia	In air before experiment	In experimental atmosphere	Change during experiment	In air after experiment	Normal before pneumonia	In air before experiment	In experimental atmosphere
			O_2	CO_2								
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
A. Normal	O_2	109	96	0.1		96	98	+2			7.37	7.30
		110	96	0.1		91	100	+9	92		7.34	7.30
		111	96	0.1		95	99	+4	94		7.37	7.36
		112	97	0.1		93	100	+7	93		7.40	7.40
								+5				
B. "	CO_2	113	19.6	9.3		93	97	+4	93		7.37	7.27
		114	22.0	9.5		92	96	+4	92		7.37	7.22
		115	21.0	9.2		95	96	+1	93		7.37	7.18
		116	20.0	9.9		94	96	+2	92		7.37	7.14
		117	19.0	9.7		93	94	+1	93		7.37	7.07
		124	20.4	10.2		93	96	+3	96		7.36	7.17
		125	22.5	9.3		96	96	0	95		7.35	7.13
		126	21.8	9.7		97	98	+1	95		7.34	7.16
		127	21.5	9.8		96	96	0	92		7.34	7.12
								+2				
C. "	$O_2 + CO_2$	121	60.0	10.4		96	100	+4	95		7.38	7.16
		122	58.0	10.1		94	100	+6	95		7.37	7.14
		123	63.0	9.1		95	100	+5	95		7.34	7.12
								+5				
D. Pneumonia	Ordinary air	128	21	0.1	94	83	79	−4	76	7.33	7.34	7.35
		137	21	0.1	92	77	82	+5	87	7.37	7.36	7.37
		141	21	0.1	97	80	80	0	82	7.37	7.35	7.36
		143	21	0.1	94	78	82	+4	86	7.33	7.35	7.36
								+1				
E. "	O_2	129	78	0.1	95	77	93	+16	92	7.34	7.39	7.36
		130	97	0.1	96	84	100	+16	82	7.38	7.37	7.36
		131	97	0.1	99	77	95	+18	75	7.35	7.36	7.34
		132	97	0.1	93	83	100	+17	87	7.36	7.33	7.33
								+17				
F. "	CO_2	138	21.5	10.0	94	58	59	+1	53	7.34	7.30	6.90
		133	21.4	10.3	96	70	70	0	61	7.36	7.36	7.05
		134	21.0	10.4	97	85	81	−4	87	7.33	7.36	6.90
		135	21.9	8.8	94	73	73	0	77	7.36	7.40	7.08
		136	21.5	10.2	96	76	86	+10	84	7.35	7.33	6.95
								+1				
G. "	$O_2 + CO_2$	145	71	12.7	96	82	95	+13	81	7.37	7.37	7.10
		146	75	10.1	90	76	99	+23	76	7.37	7.38	7.08
		142	66	9.9	98	83	99	+16	88	7.35	7.34	7.13
		139	64	10.3	98	67	90	+23	61	7.34	7.32	7.02
		140	62	11.0	93	71	80	+9	49	7.32	7.32	6.90
								+17				

id-Base Balance of Blood

		CO_2 tension					Serum $(HCO_3)_1$				
Change during experiment	In air after experiment	Normal before pneumonia	In air before experiment	In experimental atmosphere	Change during experiment	In air after experiment	Normal before pneumonia	In air before experiment	In experimental atmosphere	Change during experiment	In air after experiment
		mm. Hg	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
−0.07			34.4	42.5	+8.1			19.3	18.6	−0.7	
−0.04	7.32		33.3	36.2	+2.9	35.2		17.2	16.4	−0.8	17.1
−0.01	7.36		31.9	33.9	+2.0	33.9		17.9	18.4	+0.5	18.4
0	7.40		34.6	32.2	−2.4	31.6		21.0	19.8	−1.2	19.6
−0.03					**+2.6**					**−0.5**	
−0.10	7.35		34.2	54.2	+20.0	58.6		18.8	21.4	+2.6	20.1
−0.15	7.36		33.8	58.6	+24.8	38.4		18.7	20.0	+1.3	20.4
−0.19	7.36		35.9	64.6	+28.7	40.2		19.8	19.3	−0.5	21.8
−0.23	7.36		34.6	73.0	+38.4	34.6		18.9	18.4	−0.5	18.8
−0.30	7.34		35.0	83.7	+48.7	39.3		19.2	15.1	−4.1	19.7
−0.19	7.36		42.4	76.6	+34.2	43.7		23.2	22.6	−0.6	23.6
−0.22	7.34		40.8	79.7	+38.9	45.8		21.8	21.0	−0.8	23.7
−0.18	7.34		40.5	78.0	+37.5	45.2		21.2	22.2	+1.0	23.2
−0.22	7.33		40.7	82.5	+41.8	44.2		21.3	21.3	0	22.8
−0.20					**+34.8**					**−0.2**	
−0.22	7.37		38.8	77.0	+38.2	42.0		22.2	21.6	−0.6	23.5
−0.23	7.36		34.6	75.8	+41.2	38.4		19.9	19.9	0	20.8
−0.22	7.33		35.7	71.0	+35.3	38.1		18.6	16.8	−1.8	19.1
−0.22					**+38.2**					**−0.8**	
+0.01	7.37	39.4	35.8	34.4	−1.4	32.0	20.2	19.0	19.0	0	18.8
+0.01	7.37	35.6	30.2	34.6	+4.4	35.0	20.0	16.3	19.4	+3.1	18.9
+0.01	7.32	32.2	31.6	32.9	+1.3	34.6	17.9	16.4	17.4	+1.0	16.2
+0.01	7.35	36.5	34.0	32.6	−1.4	31.6	18.9	18.7	18.2	−0.5	17.4
+0.01					**+0.7**					**+0.9**	
−0.03	7.37	36.7	35.8	39.4	+3.6	37.0	19.2	22.2	22.0	−0.2	21.1
−0.01	7.37	44.0	43.2	45.0	+1.8	42.5	25.2	24.0	23.8	−0.2	23.6
−0.02	7.36	38.6	39.4	43.0	+3.6	40.1	20.7	21.8	22.2	+0.4	22.2
0	7.33	38.8	44.6	44.6	0	46.0	21.2	21.8	22.2	+0.4	22.8
−0.01					**+2.2**					**+0.1**	
−0.40	7.26	32.8	34.6	100.9	+66.3	36.2	17.2	15.6	9.3	−6.3	14.0
−0.31	7.36	41.6	36.5	87.2	+50.7	36.3	22.8	20.3	16.2	−4.1	20.2
−0.46	7.29	31.5	29.2	97.2	+68.0	26.4	16.0	16.2	9.0	−7.2	11.4
−0.32	7.38	35.5	33.2	87.1	+53.9	36.1	19.5	21.3	18.0	−3.3	21.1
−0.38	7.30	34.7	30.1	94.7	+64.6	35.8	18.5	14.9	10.0	−4.9	15.4
−0.37					**+60.7**					**−5.1**	
−0.27	7.37	35.3	32.7	80.4	+47.7	36.9	19.8	18.3	17.9	−0.4	20.7
−0.30	7.37	30.4	31.8	85.3	+53.5	36.8	17.1	18.5	18.0	−0.5	20.6
−0.21	7.36	37.3	35.6	78.7	+43.1	37.7	20.0	18.4	20.4	+2.0	20.8
−0.30	7.28	33.6	37.0	93.0	+56.0	45.3	17.6	18.2	16.1	−2.1	18.7
−0.42	7.34	36.3	32.6	109.8	+77.2	31.1	18.1	16.8	11.5	−5.3	16.7
−0.30					**+55.5**					**−1.3**	

tinuously circulating air. Whenever possible, the same animals which had been subjects for control experiments (Series 1) were used. After a lapse of about 2 hours, oxygen was continuously introduced into the cage until the oxygen content was above 90 per cent. The oxygen concentration was kept at this level for about 2 hours. At the end of this time, the oxygen concentration was reduced by introducing a stream of air. Two blood samples were taken an hour apart during the preliminary period in ordinary air. After the oxygen content of the cage had reached the desired level, two more blood samples were drawn, again an hour apart. Two final blood samples, at about a 1 hour interval, were drawn during the concluding control period in ordinary air. Throughout this series of experiments, the carbon dioxide was removed by recirculation of the cage gases through a soda lime tower. The composition of the cage atmosphere was checked at frequent intervals, but only the results on samples which were taken simultaneously with the drawing of blood are recorded in Table IV and Protocols 1 and 2. In all subsequent experiments, the same standard technique was followed, except that the composition of the gases breathed was modified as indicated.

The four normal dogs subjected to high oxygen atmospheres showed an average increase of 5 per cent in blood oxygen saturation from the normal of 95 per cent to almost complete saturation. The average change in pH was -0.03, in carbon dioxide tension $+2.6$ mm. of Hg, and in $(HCO_3)_1$ -0.5 mM per liter (Table IV, A). It is to be noted, therefore, that although oxygenation of the blood was increased in normal animals breathing high concentrations of oxygen, the acid-base changes remained within the normal physiological limits.

With the same standard technique, studies were made of the effect of high oxygen tensions on pneumonic dogs. The oxygen saturation in all individuals was restored to about the normal level, as was to be expected from the work of many previous investigations on animals and patients. There was an average increase in oxygen saturation of 17 per cent, which may be directly attributed to the increase in alveolar oxygen tension. Only slight variations occurred in acid-base balance. The average excursion in pH was -0.01, in carbon dioxide tension $+2.2$ mm. of Hg, and in serum bicarbonate $+0.1$ mM per liter (Table IV, E).

These experiments on pneumonic dogs indicate even more definitely than those on normal dogs that the oxygen saturation of the blood is dependent upon the oxygen tension of the alveolar air. The degree of anoxemia occurring in our cases of experimental pneumonia and the increased oxygenation following oxygen administration had no significant effect upon the state of the acid-base balance.

Series 4. Effect of Carbon Dioxide on Normal and Pneumonic Dogs—In the preceding section, it was shown that, whereas the oxygen saturation of the blood was decreased in pneumonia and restored to normal by the administration of oxygen, the acid-base balance was affected by neither the pneumonic process nor subsequent oxygen administration. The next step was to study the effect of the addition of carbon dioxide to the inspired air upon oxygen saturation and acid-base balance.

In the same standard type of experiments previously described, 10 per cent of the nitrogen in ordinary air was replaced by carbon dioxide. This concentration, which is higher than that used clinically, was chosen in order to exaggerate the smaller changes which accompany the use of lower concentrations. Normal dogs breathing this atmosphere (21 per cent oxygen, 10 per cent carbon dioxide, 69 per cent nitrogen) showed an average increase of 2 per cent in oxygen saturation (Table IV, B). That the slight increase observed was probably real was shown by the fact that the oxygen saturation returned to normal when carbon dioxide was again removed from the air of the chamber. It should be noted that the increase observed in oxygenation of the blood when the animal was breathing atmospheres high in carbon dioxide is perhaps the more remarkable because the lowered pH produced in the blood would tend to favor the dissociation of oxyhemoglobin rather than its formation.

Naturally, the acid-base balance was markedly affected by the absorption of excess carbon dioxide into the blood. This was indicated by an average increase of 34.8 mm. of Hg in carbon dioxide tension, from an average level of 37.5 to 72.3. The pH fell 0.2 unit, but the average effect upon the bicarbonate was inappreciable (Table IV, B). The detailed changes in these experiments will be discussed in a later section.

The same type of experiments repeated with pneumonic dogs resulted in an average increase of 1 per cent in oxygenation (Table IV, F). The carbon dioxide tension changed from an average of 32.7 to 93.2, or an average increase of 60.7 mm. of Hg. There was a decrease of 0.37 unit in pH and a decrease of 5.1 mM per liter in the bicarbonate content.

Series 5. Effect of Mixtures of Oxygen and Carbon Dioxide on Normal and Pneumonic Dogs—This final series of experiments was carried out with animals breathing an atmosphere with a high content of oxygen and added carbon dioxide.

The effect of the breathing of atmospheres composed of 60 per cent oxygen, 10 per cent carbon dioxide, and 30 per cent nitrogen on normal dogs was to raise the oxygenation of the blood from 95 per cent to 100 per cent. The pH decreased 0.22 unit; the carbon dioxide tension increased from 36.4 to 74.6, or 38.2 mm. of Hg; the bicarbonate decreased 0.8 mM per liter (Table IV, C).

It is interesting that the effects of oxygen and carbon dioxide combined were, in general, the additive effects of each of these gases separately. High carbon dioxide-oxygen atmospheres increased the oxygenation of both normal and pneumonic animals. It is, therefore, obvious that the addition of carbon dioxide to the inspired air does not modify the effect of oxygen alone upon the oxygenation of the blood.

The breathing of atmospheres rich in carbon dioxide and with high percentages of oxygen caused approximately the same drop in pH (about 0.2) in the serum of the normal dogs as did the breathing of air rich in carbon dioxide alone. When pneumonic dogs breathed air enriched with oxygen and carbon dioxide, there was a smaller decrease in pH (amounting to 0.30 in comparison with 0.37) than when pneumonic dogs breathed atmospheres enriched only with carbon dioxide. The increase in the carbon dioxide tension of the normal dogs breathing 60 per cent oxygen and 9 to 10 per cent carbon dioxide was approximately the same as that observed when they were breathing 21 per cent oxygen and 9 to 10 per cent carbon dioxide. This amounted to an average of 38.2 mm. of mercury in comparison with 34.8 for atmospheres high in carbon dioxide alone. The pneumonic animals, breathing oxygen plus carbon dioxide, showed an average increase of the carbon dioxide tension which was not significantly different from that observed when they were in an atmosphere of air plus carbon dioxide, 55.5 mm. compared with 60.7 mm. These figures confirm the previous findings that the carbon dioxide tension of the blood of the pneumonic dogs increased more for a given increase in carbon dioxide tension of the inspired air than did that of normal animals.

When the pneumonic dogs breathed atmospheres high in carbon dioxide, the decrease in bicarbonate concentration was 5.1 mM per liter. The same type of dogs breathing air high in both oxygen and carbon dioxide showed a decrease of only 1.3 mM per liter.

Acid-Base Paths of Displacement and Recovery

The acid-base balance of the blood was not appreciably displaced from normal in the pneumonic dogs whether they were breathing air or oxygen. When CO_2 was added to the inspired air or oxygen, a pronounced acid-base displacement occurred. Illustrative experiments showing the direction and extent of the displacement have been plotted in Fig. 2.

In the lower right-hand corner of Fig. 2, there is included a small acid-base diagram drawn according to that described by Shock and Hastings (24). It is drawn to the same scale as that of the rest of Fig. 2. The hexagon represents the normal acid-base area which includes the data of our normal dogs. The arrows indicate the directions in which the acid-base balance is usually displaced in respiratory acidosis and alkalosis, and in metabolic acidosis and alkalosis.

Interest in the acid-base paths centers around the direction and extent of displacement from normal. The data chosen for illustration include the acid-base paths before, during, and following the administration of CO_2 in air to a normal dog and a pneumonic dog (Fig. 2, *A*); and of CO_2 in oxygen-enriched air to a normal dog and a pneumonic dog (Fig. 2, *B*).

It is at once apparent that the direction of displacement is essentially that of respiratory acidosis in all four cases. There are, however, characteristics of the four curves (Fig. 2) which require special mention. (1) The direction of initial displacement of normal dogs, breathing air plus CO_2, and oxygen plus CO_2, closely approaches the line representing the titration of blood with CO_2. (Compare with the line labeled "Respiratory acidosis.") Subsequent determinations, made at hourly intervals while the animals were in the high CO_2 atmosphere, showed further acid-base displacement in a direction which indicated that a change characteristic of metabolic alkalosis was superimposed upon the respiratory acidosis. Such a change could be accounted for either by a de-

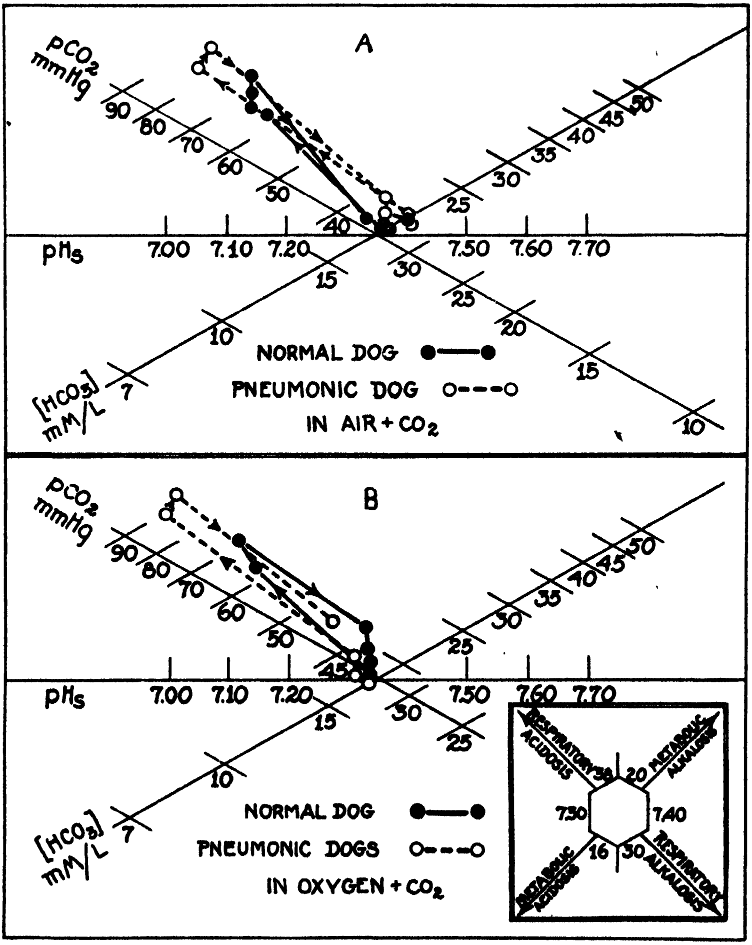


FIG. 2. The paths of acid-base displacement and recovery following the administration of CO_2 in air and CO_2 in oxygen to normal and pneumonic dogs. The arrows on the chart in the lower right-hand corner indicate the direction characteristic of different types of acid-base disturbance. The hexagon represents the normal acid-base area for dogs.

crease in the concentration of fixed acid or an increase in the concentration of fixed base. Physiologically, it represents compensation by metabolic processes for the respiratory acidosis following CO_2 administration, just as compensation for metabolic acidosis is brought about by respiratory activity. Following the restoration of the atmosphere to air without CO_2, the acid-base balance returned to normal values. (2) The direction of initial displacement of pneumonic dogs did not follow the line of the titration of blood with CO_2 as closely as did that of the normal dogs. Its direction corresponded to that which it would have been if a component characteristic of metabolic acidosis were superimposed upon the respiratory acidosis. A subsequent change occurred after the animals had been in the high CO_2 atmosphere, which was similar to that which was observed in the experiments on normal dogs; namely, a change in the acid-base balance characteristic of metabolic alkalosis. This change can also be regarded as a physiological compensation for the severe respiratory acidosis. (3) The extent of the acid-base displacement following CO_2 administration was considerably greater in the pneumonic dogs than in the normal dogs. This is due to the inability of the pneumonic dogs to provide adequate exchange of carbon dioxide in the lungs. When the tension of CO_2 in the atmosphere was between 60 and 70 mm. of Hg, the CO_2 tension of the arterial blood of the normal dogs was only about 5 mm. above that of the inspired air (Table V). In the pneumonic dogs, however, the CO_2 tension of the blood was

TABLE V

Comparison of CO_2 Tension of Inspired Air and Arterial Blood of Normal and Pneumonic Dogs in High CO_2 Atmosphere

Two examples are given for each experimental condition.

Dogs		CO_2 tension			
		In air + CO_2		In O_2 + CO_2	
		mm. Hg	*mm. Hg*	*mm. Hg*	*mm. Hg*
Normal	Blood	64.0	67.6	64.0	71.0
	Inspired air	57.5	61.1	59.6	64.4
	Difference	6.5	6.5	4.4	6.6
Pneumonic	Blood	86.0	88.3	92.0	93.0
	Inspired air	59.0	62.4	63.2	73.2
	Difference	27.0	25.9	28.8	19.8

approximately 25 mm. above that of the inspired air. This great difference persisted even though the inspired air was enriched with oxygen. It is thus evident that the ability of pneumonic dogs to eliminate CO_2 from the blood, in the presence of a high CO_2 tension of the inspired air, is much less than that of normal animals.

DISCUSSION

Since about 8 years have elapsed since Henderson *et al.* (11) have suggested the application of carbon dioxide in the treatment of pneumonia, interest naturally centers on the clinical results obtained by those who have tried this method.

Hanson and Calhoun (25) tried the effect of atmospheres containing 5 per cent carbon dioxide and 95 per cent oxygen on patients who had been ill with lobar pneumonia for no longer than 3 days. These patients were allowed to breathe this mixture for periods of 10 minutes twice a day. x-Ray changes and the clinical course of the disease were carefully studied. The authors state, "Our investigations indicate that with the dosage and apparatus used, early pneumonia could not be aborted. So far as we could determine, inhalation of carbon dioxide and oxygen in this concentration is accompanied by no harmful effects."

Alison (26) claimed that the disease could be arrested if treatment were begun in the first 36 hours. In his opinion, the patients "were more comfortable and appeared to progress better" with carbon dioxide added than with oxygen alone.

Hilton (27) tried the effect of 3 per cent carbon dioxide without added oxygen on cases of lobar pneumonia. The gas was administered with a loose face-mask, and the examinations of the breathing and the blood were repeated 15 minutes later. He found that arterial anoxemia is unrelieved after carbon dioxide administration in those quantities, in spite of the fact that the tidal air is considerably increased. He concludes that "in the absence of respiratory failure, as evinced by shallow breathing, the clinical benefits of CO_2 administration in air are not sufficiently demonstrated by these observations to warrant its routine use in lobar pneumonia."

Gunther and Blond (28) presented a clinical study of the use of carbon dioxide in early cases of both broncho- and lobar pneumonia which was carried over a period of 5 years. Their results appear favorable, the authors reporting a mortality of 4 per cent in the group receiving early inhalation treatment with 5 per cent carbon dioxide in oxygen, in comparison with 17 per cent in the group that received no carbon dioxide treatment.

It would seem, therefore, that from physiological considerations, animal experimentation, and clinical studies the value of carbon dioxide in pneumonia is still open to question. Because of the high concentrations of carbon dioxide employed in our experiments, and the fact that experimental bronchopneumonia in dogs is quite different from pneumonia in humans, our results cannot be directly applied to the clinical use of carbon dioxide. They do suggest, however, that carbon dioxide in concentrations sufficiently great to stimulate respiration may well be used with caution.

Carbon dioxide is a respiratory stimulant and as such serves a definite purpose in the treatment of asphyxia, carbon monoxide poisoning, and other conditions in which the respiration is depressed. It may even have its place in the treatment of postoperative collapse. In the absence of evidence that the CO_2 tension of the arterial blood is increased in pneumonia, it may be questioned whether there is significant depression of the respiratory center. Since oxygen alone achieves the desired reoxygenation of the blood when this is possible, the addition of carbon dioxide may be of doubtful benefit. In addition, the excessively rapid respirations induced by carbon dioxide are an added burden to an already overburdened respiratory and cardiovascular system.

SUMMARY

1. Experimental bronchopneumonia was produced in dogs by the intrabronchial injection of 1 per cent hydrochloric acid.

2. Although the pneumonia was accompanied by pronounced anoxemia, no significant change in the acid-base balance of the arterial blood was observed.

3. Oxygen-enriched atmosphere increased the oxygen saturation of the arterial blood of normal dogs slightly, and pneumonic dogs greatly, without alteration in the acid-base balance.

4. Addition of carbon dioxide to the inspired air caused a slight increase in the arterial saturation of both normal and pneumonic dogs.

5. Addition of carbon dioxide to the inspired air was accompanied by a change in the acid-base balance of the blood indicative of respiratory acidosis. The respiratory acidosis was more severe in the pneumonic dogs than in the normal dogs. Some metabolic acidosis was observed in the pneumonic dogs breathing this atmosphere.

6. Oxygen-enriched atmosphere, containing carbon dioxide, was accompanied by (*a*) increased oxygenation of the blood of normal and pneumonic dogs, (*b*) respiratory acidosis which was greater in the pneumonic than in the normal dogs.

7. Both normal and pneumonic animals, kept in the high CO_2 atmosphere, showed a change in their acid-base balance indicative of physiological compensation.

8. Evidence is presented illustrating the decreased ability of pneumonic dogs to eliminate CO_2.

9. The implication of these observations for the use of carbon dioxide with oxygen in the treatment of pneumonia is discussed.

BIBLIOGRAPHY

1. Haldane, J. S., *Brit. Med. J.*, **1**, 181 (1917).
2. Barcroft, J., Cooke, A., Hartridge, H., Parsons, T. R., and Parsons, W., *J. Physiol.*, **53**, 450 (1920).
3. Stadie, W. C., *J. Exp. Med.*, **30**, 215 (1919); **35**, 337 (1922).
4. Meakins, J. C., *J. Path. and Bact.*, **24**, 87 (1921).
5. Davies, H. W., and Gilchrist, A. R., *Edinburgh Med. J.*, **32**, 225 (1925).
6. Binger, C. A. L., *J. Clin. Inv.*, **6**, 203 (1928–29).
7. Barach, A. L., *Arch. Int. Med.*, **37**, 186 (1926).
8. Boothby, W. M., and Haines, S. F., *Tr. Assn. Am. Physn.*, **42**, 287 (1927).
9. Meakins, J. C., *Arch. Int. Med.*, **25**, 1 (1920).
10. Lundsgaard, C., and Van Slyke, D. D., Cyanosis, Baltimore (1922).
11. Henderson, Y., Haggard, H. W., Coryllos, P. N., Birnbaum, G. L., and Radloff, E. M., *Arch. Int. Med.*, **45**, 72 (1930).
12. Henderson, Y., *Am. J. Physiol.*, **21**, 126 (1908).
13. Henderson, Y., and Haggard, H. W., *J. Pharmacol. and Exp. Therap.*, **16**, 11 (1920).
14. Henderson, Y., and Haggard, H. W., *J. Am. Med. Assn.*, **79**, 1137 (1922).
15. Coryllos, P. N., and Birnbaum, G. L., *Arch. Surg.*, **16**, 501 (1928); *Am. J. Roentgenol.*, **22**, 401 (1929); *Arch. Surg.*, **18**, 190 (1929).
16. Henderson, Y., and Haggard, H. W., *J. Am. Med. Assn.*, **92**, 434 (1929).
17. Henderson, Y., Coryllos, P. N., Haggard, H. W., Birnbaum, G. L., and Radloff, E. M., *Science*, **69**, 502 (1929); *Am. J. Physiol.*, **90**, 317 (1929).

18. Henderson, Y., Coryllos, P. N., Holmes, G. W., and Scott, W. J. M., *J. Am. Med. Assn.*, **93**, 96 (1929).
19. Henderson, Y., *New England J. Med.*, **206**, 151 (1932).
20. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
21. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **61**, 695 (1924).
22. Hastings, A. B., Neill, J. M., Morgan, H. J., and Binger, C. A. L., *J. Clin. Inv.*, **1**, 25 (1924).
23. Binger, C. A. L., Hastings, A. B., and Sendroy, J., Jr., *J. Exp. Med.*, **45**, 1081 (1927).
24. Shock, N. W., and Hastings, A. B., *J. Biol. Chem.*, **112**, 239 (1935–36).
25. Hanson, J. F., and Calhoun, A. W., *Arch. Int. Med.*, **50**, 269 (1932).
26. Alison, J. F., *South. Med. J.*, **25**, 386 (1932).
27. Hilton, R., *Brit. Med. J.*, **1**, 418 (1934).
28. Gunther, L., and Blond, H. H., *Am. J. Med. Sc.*, **193**, 525 (1937).

Protocol 1

Experiments on Normal Dogs

Time	Respiration	Air in cage		O_2 content	O_2 capacity	O_2 saturation	pH_s	Total $(CO_2)_s$	$(HCO_3)_s$	$(HCO_3)_1$	$\Delta(HCO_3)_1$	pCO_2	ΔpCO_2
		O_2	CO_2										
In air. Experiment C108; Dog 89; weight 25 pounds													
		per cent	*per cent*	*vol. per cent*	*vol. per cent*	*per cent*		*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mm. Hg*
10.15 a.m.	20	21	0.1	17.5	19.5	90	7.35	18.39	17.41	17.41		32.6	
11.10 "	18	21	0.1	17.7	19.4	91	7.35	18.28	17.31	17.31	−0.10	32.2	−0.4
12.00 "	18	21	0.1	17.6	19.4	91	7.34	18.78	17.76	17.52	+0.11	33.9	+1.3
1.00 p.m.	14	21	0.1	17.6	19.4	91	7.35	18.48	17.50	17.50	+0.09	32.6	0
1.50 "	19	21	0.1	17.4	19.1	91	7.35	19.0	17.99	17.99	+0.58	33.6	+1.0
2.45 "	18	21	0.1	17.2	18.9	91	7.35	19.71	18.66	18.66	+1.25	34.9	+2.3
3.45 "	22	21	0.1	17.2	18.6	92	7.35	19.06	18.05	18.05	+0.64	33.6	+1.0
4.30 "		21	0.1	16.7	17.9	93	7.35	18.28	17.31	17.31	−0.10	32.3	−0.3
In atmosphere enriched with O_2. Experiment C111; Dog 89; weight 25 pounds													
10.10 a.m.	15	21	0.1	16.9	17.9	94	7.37	18.92	17.96	17.96		31.9	
11.05 "	15	21	0.1	17.2	17.9	96	7.37	18.83	17.87	17.87	−0.09	31.9	0
12.35 p.m.	14	82	0.1	17.2	17.2	100	7.35	18.70	17.70	17.23	−0.73	33.2	+1.3
1.30 "		90	0.1	17.3	17.3	100	7.36	18.39	17.43	17.18	−0.78	31.9	0
2.35 "	13	96	0.1	17.3	17.5	99	7.36	19.64	18.62	18.38	+0.42	33.9	+2.0
3.35 "	12	24	0.1				7.34	19.82	18.74	18.01	+0.05	35.9	+4.0
3.37 "				16.3	16.3	100	7.34	19.96	18.87	18.15	+0.19	36.2	+4.3
4.20 "	14	21	0.1	15.8	16.8	94	7.36	19.64	18.62	18.38	+0.42	33.9	+2.0

In air enriched with CO_2. Experiment C116; Dog 89; weight 25 pounds												
10.00 a.m.	15	21	0.1	19.3	20.5	94	7.37	20.0	18.98	18.98		33.9	
11.05 "	15	21	0.1	18.8	20.0	94	7.36	20.04	18.98	18.75	−0.23	35.3	+1.4
1.15 p.m.	40	21	8.1	18.8	19.4	97	7.17	24.55	22.62	17.65	−1.33	64.0	+30.1
2.20 "	42	20	8.6	18.0	18.8	96	7.14	24.33	22.30	16.60	−2.38	67.6	+33.7
3.20 "	43	20	9.2	17.9	18.7	96	7.14	25.31	23.19	17.52	−1.46	70.3	+36.4
4.15 "		20	9.9	17.9	18.7	96	7.14	26.29	24.09	18.35	−0.63	73.0	+39.1
5.20 "	17	21	0.3	17.7	19.4	91	7.33	20.09	18.97	17.98	−1.0	37.1	+3.2
6.05 "	17	21	0.1	17.2	18.7	92	7.36	20.0	18.96	18.75	−0.23	34.6	+0.7
In atmosphere enriched with O_2 and CO_2. Experiment C123; Dog 89; weight 27 pounds													
10.10 a.m.	13	21	0.1	19.2	20.2	95	7.34	20.0	18.91	18.91		36.2	
11.15 "	12	21	0.1	19.2	20.2	95	7.34	19.42	18.36	18.36	−0.55	35.2	−1.0
1.15 p.m.	23	58	8.4	20.4	20.4	100	7.15	23.51	21.59	16.97	−1.94	64.0	+27.8
2.25 "	30	63	9.1	20.6	20.6	100	7.12	24.40	22.27	16.81	−2.10	71.0	+34.8
4.15 "	19	21	0.3	18.7	19.5	96	7.33	22.48	21.23	21.0	+2.09	41.7	+5.5
4.50 "		21	0.1	18.5	19.5	95	7.33	20.58	19.43	19.13	+0.22	38.1	+1.9

Protocol 2

Experiments on Pneumonic Dogs

Time	Respiration	Air in cage		O_2 content	O_2 capacity	O_2 saturation	pH_s	Total $(CO_2)_s$	$(HCO_3)_s$	$(HCO_3)_1$	$\Delta(HCO_3)_1$	pCO_2	ΔpCO_2
		O_2	CO_2										
In air. Experiment C141; Dog 154; weight 31 pounds													
		per cent	*per cent*	*vol. per cent*	*vol. per cent*	*per cent*		*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mm. Hg*	*mm. Hg*
*		21	0.1	25.0	25.9	97	7.37	18.87	17.90	17.9		32.2	
10.45 a.m.	74	21	0.1	23.9	29.7	80	7.35	17.82	16.87	16.38	−1.52	31.6	−0.6
1.15 p.m.	62	21	0.1	22.0	26.9	82	7.35	19.1	18.08	17.58	−0.32	33.9	+1.7
2.50 "	59	21	0.1	20.4	26.2	78	7.36	18.44	17.48	17.25	−0.65	32.0	−0.2
4.50 "	56	21	0.1	21.2	25.8	82	7.32	18.35	17.31	16.16	−1.74	34.6	+2.4
In atmosphere enriched with O_2. Experiment C131; Dog 120; weight 22 pounds													
*	18	21	0.1	23.8	24.4	99	7.35	21.83	20.67	20.67		38.6	
10.05 a.m.	63	21	0.1	21.6	28.1	77	7.36	22.77	21.58	21.80	+1.13	39.4	+0.8
11.40 "	60	95	0.1	25.3	26.8	94	7.34	22.63	21.40	21.15	+0.48	40.9	+2.3
1.05 p.m.	64	97	0.1	24.6	25.8	95	7.34	23.79	22.50	22.23	+1.56	43.0	+4.4
2.30 "	64	24	0.1	21.2	25.4	83	7.35	23.97	22.69	22.69	+2.03	42.4	+3.8
4.10 "	68	21	0.1	17.9	24.0	75	7.36	23.15	21.94	22.15	+1.48	40.1	+1.5
In atmosphere enriched with CO_2. Experiment C135; Dog 118; weight 25 pounds													
*		21	0.1	21.3	22.6	94	7.36	20.54	19.47	19.47		35.5	
9.40 a.m.	47	21	0.1	14.7	20.6	71	7.41	21.07	20.09	21.26	+1.79	32.7	−2.8
10.45 "	51	21	0.1	16.3	21.5	76	7.40	21.27	20.25	21.29	+1.82	33.8	−1.7
12.40 p.m.	94	20.7	8.3	15.8	20.8	76	7.05	25.71	23.12	16.19	−3.28	86.0	+50.5
2.10 "	90	21.9	8.8	13.8	19.0	73	7.08	27.50	24.84	18.30	−1.17	88.3	+52.8
3.30 "	46	21	0.1	13.7	18.2	75	7.36	22.12	20.96	20.97	+1.50	38.3	+2.8
4.35 "	42	21	0.1	13.4	16.8	80	7.40	21.41	20.39	21.30	+1.83	34.0	−1.5

In atmosphere enriched with O_2 and CO_2. Experiment C139;* Dog 152

*		21	0.1	23.5	23.9	98	7.34	18.57	17.56	17.56		33.6	
10.05 a.m.	63	21	0.1	16.7	24.7	67	7.32	18.57	17.52	17.10	−0.46	35.1	+1.50
11.25 "		21	0.1	16.7	24.7	67	7.32	20.13	18.99	18.50	+0.94	38.0	+4.4
1.25 p.m.	105	60	8.9	20.2	24.1	84	7.00	24.21	21.57	13.9	−3.66	90.2	+56.6
3.00 "	85	64	10.3	20.2	22.5	90	7.02	26.07	23.27	16.10	−1.46	93.0	+59.4
4.40 "	78	21	0.1	13.3	21.9	61	7.28	22.01	20.65	18.72	+1.16	45.3	+11.7

---

* A normal experiment was run before anoxemia was induced. The present experiment was carried out on the following day.

THE DISTRIBUTION OF BODY WATER IN SKELETAL MUSCLE IN DOGS WITH IMPAIRED RENAL FUNCTION

BY LILLIAN EICHELBERGER

(From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago)

(Received for publication, January 3, 1939)

In a previous paper (1), a study of the extra- and intracellular phase volumes of skeletal muscle in normal dogs, before and after an experimental increase in the total body water, was presented. Data for the normal organism having been established, it was desirable to investigate changes in water and electrolyte distribution in skeletal muscle in the organism with derangement of renal function. The present study is concerned with dogs with experimental hydronephrosis. Data will be presented on the phase volumes of skeletal muscle of dogs in chronic and progressive hydronephrosis, both previous to and following an increase in total body water as produced by the intravenous injection of slightly alkaline isotonic salt solutions. These data will be compared with those on normal animals and animals in acute uremia, which may be regarded as controls.

EXPERIMENTAL

Physiological Procedures—The dogs used in these experiments were maintained in metabolism cages throughout the long period of experimentation. Daily charts of body weight, food and water intake, and urine volume, specific gravity, and protein concentration were made.

Detailed methods for the production and diagnosis of experimental hydronephrosis have been given in a previous paper (2). Briefly, the procedure is as follows: The kidney was brought outside of the split muscle layers through an incision about 3 cm. below and parallel to the left costal margin. The ureter was partially constricted about 4 cm. from the ureteropelvic junction and the kidney replaced. A nephrectomy was performed on the opposite side 10 to 14 days later, thus leaving the single hydronephrotic kidney with increased intraureteral and intrapelvic pressure to carry on all renal excretion.

The hydronephrotic animals were classified into two groups, as previously described (2). Group I, the *chronic*, included animals in which no physical symptoms developed, although intravenous pyelograms showed the disappearance of the calyces of the kidney without further progression over a period of months; a considerable amount of functioning renal tissue remained when the animal was sacrificed, and chemical studies of the blood serum showed no significant deviation from the normal. The animal was in a non-uremic stage of hydronephrosis at the time of the final injection experiment.

Group II, the *progressive* group, included the animals which developed signs and symptoms of severe renal involvement. The greater part of the renal tissue was destroyed and therefore urographic shadows could not be obtained. These animals were in the uremic stage of hydronephrosis when the final injection experiments were carried out, and blood studies showed marked acidosis, retention of non-protein nitrogen, and increased calcium and inorganic phosphorus concentrations unaccompanied by any change in total protein concentration but accompanied by an increased albumin to globulin ratio, as previously reported (2).

The acute uremia was produced as follows: The ureter of one kidney was ligated. The kidney on the opposite side was removed 10 to 14 days later, thus leaving the animal with one non-functioning hydronephrotic kidney. The animal developed acute uremia within 3 to 5 days. The injection experiment was carried out before the animal began to vomit or to develop diarrhea. The dogs always refused food after the 1st day following the nephrectomy, although they continued to drink water.

For the injection experiments, all dogs were anesthetized with sodium barbital, as in the experiments on normal dogs (1). The blood pressure was continuously recorded from the carotid artery throughout the experiment. A cannula was introduced into the femoral artery, 30 cc. of blood were taken under oil, and one of the rectus abdominis muscles was removed for the control (initial) analyses.

The animals received an intravenous injection of 170 cc. per kilo of body weight of an alkaline isotonic salt solution consisting of 114 mM of NaCl + 40 mM of $NaHCO_3$. This slightly akaline solution was used because animals in uremia have a marked acidosis and it was hoped that they might thus be brought into normal acid-base equilibrium. The injection of the solution (warmed to 38°), which was given by gravity, required 30 to 40 minutes. 30 minutes were then allowed to elapse before the remaining rectus abdominis muscle was removed and a second sample of blood taken for the final analyses. The peritoneal fluid was then removed as completely as possible and its volume measured. The urine was collected continuously during the injection by an indwelling catheter.

Chemical Methods—The following determinations were made on the serum: pH, total CO_2, water, chloride, sodium, potassium, total protein, non-protein nitrogen, and in some cases albumin to globulin ratios, calcium, and inorganic phosphorus; on the muscle, water, chloride, sodium, potassium, and total neutral fat; on the peritoneal fluid, non-protein nitrogen, total protein, chloride, and sodium; on the urine, chloride and sodium.

All analyses of serum, peritoneal fluid, and urine were made in duplicate; all muscle analyses in quadruplicate. All muscle analyses were corrected for neutral fat. The technique for the removal and treatment of the blood and muscle was the same as that described in previous papers. The chemical methods were the same, except that total proteins were determined by the micro-Kjeldahl method of Campbell and Hanna (3). The distillation of the ammonia was carried out in the Goebel modification of the Pregl micro-Kjeldahl distillation apparatus (4). The proteins were estimated by multiplying by 6.25 the total nitrogen corrected for non-protein nitrogen.

The albumin and globulin were determined by the method of Howe (5) with the Robinson, Price, and Hogden modifications (6). Total calcium determinations were made by the method of Kramer and Tisdall (7) as modified by Clark and Collip (8), and inorganic phosphorus was determined by the Fiske and Subbarow method (9).

The details for the determination of sodium and potassium in the muscle were as follows: 7 to 8 gm. of fresh minced muscle were weighed into a 50 cc. silica beaker or a platinum dish and

4 cc. of 4 N H_2SO_4 were added. The beaker was heated in a 110° oven overnight and then overnight in a 500° muffle furnace. To the residue, 5 cc. of N HCl were added and the contents transferred quantitatively to a 50 cc. volumetric flask, with use of hot water. The contents were then cooled and made to volume. For potassium determinations, 1 cc. aliquots of this solution were placed in 10 cc. beakers and evaporated to dryness. The determination of the potassium was carried out as described by Shohl and Bennett (10). Some modifications have been given in a previous paper (2). For sodium determinations on the remaining solution 0.5 gm. of solid calcium hydroxide was added and the contents allowed to stand with intermittent shaking for 30 minutes. The mixture was then centrifuged and 20 cc. aliquots of the centrifugate were placed in 50 cc. beakers containing 2 cc. of N HCl. The contents were evaporated to approximately 2 cc. and the precipitation of sodium zinc uranium acetate was then carried out by the Butler-Tuthill method (11).

Calculations of Extra- and Intracellular Phases

From the analytical data and from the fact that all of the chloride of the skeletal muscle is confined to the reticular and collagenous connective tissue framework, dissolved in the tissue fluid which infiltrates and permeates the interstitial tissue (12), the relative proportions of extra- and intracellular phases of skeletal muscle in chronic and progressive hydronephrosis and in acute uremia—both preceding and following the injection of large volumes of fluid—were calculated by means of the equations presented in a preceding paper (1). Briefly, the amounts of extracellular phase (F) in gm. per kilo of muscle were calculated from the equation

$$(F) = \frac{(\mathrm{Cl})_M \times (\mathrm{H_2O})_s \times 1000}{1.04 \times (\mathrm{Cl})_s}$$

in which the subscripts M and s represent muscle and serum respectively. From the values for (F), the volume changes produced in the extra- and intracellular phases of 1 kilo of original muscle were estimated.

Results

In Tables I to III the results, both preceding (initial) and following (final) the increase in total body water, are presented in detail. Control data on dogs with normal kidneys have been reported in a previous paper (1), (Table IV); in these animals, the increase in total body water was produced by the intravenous injection of 170 cc. per kilo of body weight of a salt solution containing 25 mM of $NaHCO_3$ + 129 mM of NaCl per liter. The increase in total body water in animals with kidney damage was produced by the intravenous injection of 170 cc. per kilo of body weight of a slightly alkaline isotonic salt solution containing 40 mM of $NaHCO_3$ + 114 mM of NaCl. In all groups of animals, injection experiments were carried out indentically in order that the physical factors influencing the flow of liquid from vascular system to muscle—such as colloidal osmotic pressure, blood pressure, and area of filtering surface—might be common to all of the experiments. Since all experiments were carried out in this parallel manner, comparison of muscle phase changes in animals with kidney damage could be made with those in animals with normal kidneys.

In all the experiments on dogs with normal kidneys and dogs with kidney damage, there was little change in the initial mean blood pressure and the blood pressure during and following the injection.

Animals with Normal Kidneys—Before injection, the normal extracellular phase of 1 kilo of skeletal muscle amounted to 173 gm., with a standard deviation (σ) of ±22 gm., and the intracellular phase amounted to 827 gm., σ ±22. Following the injection, the changes produced in the bulk of 1 kilo of muscle amounted to an average of 48 gm., σ ±10, per kilo of original muscle, consisting of a 36 gm. increase in extracellular phase and a 12 gm. increase (a negligible amount) in intracellular phase. It was concluded, therefore, that the experimental edema produced in the normal organism maintained in normal acid-base balance, following an increase in total body water, was entirely accounted for by an increase in the extracellular phase of the skeletal muscle.

Animals with Kidney Damage. Acute Uremia—Data for three typical experiments on animals in acute uremia are presented in Table I. Preceding the acute edema, the blood serum picture in acute uremia showed marked acidosis and accumulation of

TABLE I

Animals in Acute Uremia with No Kidney Function; Changes in Serum and Muscle after Injection of Alkaline Isotonic Solutions

Solution, 114 mM of NaCl + 40 mM of $NaHCO_3$. All muscle values are corrected for total neutral fat.

	pH	CO_2	Non-protein N	Protein	H_2O	Cl	Na		Ca	(*F*)
		mM per l.	*gm. per kg.*	*gm. per kg.*	*gm. per gm.*	*m.eq. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*mM per kg.*	
Dog 70; weight 23 kilos; 3400 cc. injected; duration of anuria, 4 days; peritoneal fluid, 75 cc.										
Serum. Initial	7.28	13.97	2.23	57.9	0.9116	100.8	138	8.67	2.64	
Final	7.26	22.67	1.98	31.6	0.9396	107.4	144	5.25	1.87	
Muscle. Initial					0.7770	24.75	31.87			214
Final					0.7849	27.60	38.76			232
Peritoneal fluid, per liter			1.99	18.4		112.7				
Dog 71; weight, 20.5 kilos; 3400 cc. injected; duration of anuria, 3 days; peritoneal fluid, 115 cc.										
Serum. Initial	7.29	16.60	1.97	64.1	0.9142	95.10	124	7.97	2.67	
Final	7.42	21.50	1.87	40.2	0.9385	101.8	133	5.37	1.98	
Muscle. Initial					0.7718	21.03	26.68			194
Final					0.7916	25.03	33.70			222
Peritoneal fluid, per liter						109.3	140			
Dog 73; weight, 19 kilos; 3000 cc. injected; duration of anuria, 4 days; peritoneal fluid, 500 cc.										
Serum. Initial	7.32	27.77	1.64	56.4	0.9177	93.64	138	5.51	2.63	
Final	7.42	31.32	1.35	33.8	0.9457	102.7	143	4.57	2.03	
Muscle. Initial					0.7615	18.40	30.85			173
Final					0.7734	24.60	39.07			218
Peritoneal fluid, per liter			1.48	18.3		110.4	155			

non-protein nitrogen constituents, inorganic phosphorus, and potassium. Before the injection, the extracellular phase of 1 kilo of muscle amounted to 190 gm., σ ±14, and the intracellular phase to 810 gm., σ ±14. The injection of 170 cc. per kilo of body weight of a solution containing 40 mM of $NaHCO_3$ + 114 mM of NaCl brought the body into normal acid-base equilibrium. There was an average increase in the total muscle, following injection, of 55 gm., σ ±21, of which 37 gm., σ ±15, represent an increase in extracellular phase and 18 gm., σ ±14, an increase in intracellular phase.

Chronic Non-Uremic Hydronephrosis—The analytical data, both preceding and following acute experimental edema, for three animals in chronic non-uremic hydronephrosis are presented in Table II. In all animals in this group, four in number, the concentrations of blood and skeletal muscle constituents did not differ from those observed in normal dogs. Before injection the extracellular phase amounted to 160 gm., σ ±15, per kilo, and the intracellular phase to 840 gm., σ ±15. The increase in total body water led to a gain in the total skeletal muscle averaging 66 gm., σ ±14, per kilo, of which 22 gm., σ ±16, represented an increase in intracellular phase and 44 gm., σ ±18, an increase in extracellular phase.

Uremic Stage of Progressive Hydronephrosis—Three representative experiments of the nine actually considered on animals in the uremic stage of progressive hydronephrosis are presented in Table III. A number of animals should be considered, because in this advanced stage of disease there may be attendant complications which may interfere with critical evaluation of the data.

The blood changes previously noted as characteristic of this stage of the disease were found; that is, severe acidosis, with retention of non-protein nitrogen and inorganic phosphates. Before injection, the extracellular phase amounted to 201 gm., σ ±29, and the intracellular phase to 799 gm., σ ±29. After the injection, the increase in the total skeletal muscle averaged 81 gm. per kilo, σ ±15, of which 69 gm., σ ±17, represented an increase in the extracellular phase and 10 gm., σ ±13, (a negligible amount) increase in intracellular phase.

DISCUSSION

The statistical method was applied to the data for the phase volumes, before and after the injections, of skeletal muscle from dogs with normal kidneys and from dogs in acute uremia and in

TABLE II

Animals in Chronic Non-Uremic Hydronephrosis; Changes in Serum and Muscle After Injection of Alkaline Isotonic Solutions*

Solution, 114 mM of NaCl + 40 mM of $NaHCO_3$. All muscle values are corrected for total neutral fat.

	pH	CO_2	Non-protein N	Protein	H_2O	Cl	Na	K	Ca	Inorganic P	(*F*)
Dog 90; weight 23 kilos; 3900 cc. injected; duration of disease, 12 mos.; peritoneal fluid, 165 cc.; urine, 610 cc.; blood pressure; control, 160 mm.; after injection, 156 mm.											
		mM per l.	*gm. per kg.*	*gm. per kg.*	*gm. per gm.*	*m.eq. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*mM per kg.*	*mM per kg.*	
Serum. Initial	7.39	27.42	0.52	60.4	0.9189	105.2	138	4.27	2.79	1.25	
Final	7.42	32.90	0.44	37.4	0.9475	111.5	147	3.2	1.58	0.98	
Muscle. Initial					0.7681	17.90	28.6	94.6			151
Final					0.7813	22.30	33.6	91.8			188
Peritoneal fluid, per liter			0.43	16.05		115.2	150				
Urine, excreted						49.4	56.7				
Dog 91; weight 20 kilos; 3400 cc. injected; duration of disease, 9 mos.; peritoneal fluid, 260 cc.; urine, 855 cc.; blood pressure, control, 130 mm.; after injection, 130 mm.											
Serum. Initial	7.36	23.92	0.48	59.5	0.9195	106.9	137	3.27	2.49	1.38	
Final	7.49	27.00	0.37	40.0	0.9443	107.8	138	2.64	1.65	0.87	
Muscle. Initial					0.7636	17.38	26.65	86.3			144
Final					0.7811	23.72	36.40	78.7			200
Peritoneal fluid, per liter			0.35	16.8		115.0	145				
Urine, excreted						91.8	105				

Dog 92; weight 24 kilos; 4000 cc. injected; duration of disease, 17 mos.; peritoneal fluid, 328 cc.; urine, 700 cc.; blood pressure, control, 120 mm.; after injection, 122 mm.

Serum. Initial	7.42	21.60	0.38	64.2	0.9215	104.6	142	3.85	2.52	1.11	
Final	7.48	29.70	0.23	34.4	0.9498	110.0	146	3.36	1.53	0.86	
Muscle. Initial					0.7642	19.10	32.4	96.0			162
Final					0.7822	21.84	38.9	91.0			181
Peritoneal fluid, per liter			0.34	7.0		118.5	151				
Urine, excreted						100	118				

---

* *Histological Findings*—Dr. Louis Leiter has kindly studied the microscopic sections from these kidneys. His findings are summarized as follows: Considerable variation in sections depending undoubtedly on the degree of compression of the parenchyma in different areas. In general, focal mononuclear cell infiltration in medulla, less in cortex; dilated glomerular capsular spaces and fluid in tubules; edema of medulla; interstitial edema in cortex. No fibrosis in one section; in another, scarring of lower medulla with focal subcapsular scars in cortex.

TABLE III

Animals in Progressive Uremic Hydronephrosis; Changes in Serum and Muscle after Injection of Alkaline Isotonic Solutions*

Solution, 114 mM of NaCl + 40 mM of $NaHCO_3$. All muscle values are corrected for total neutral fat.

	pH	CO_2	Non-protein N	Protein	Albumin to globulin ratio	H_2O	Cl	Na	K	Ca	Inorganic P	(*F*)
Dog 95; weight 12.8 kilos; 1800 cc. injected; duration of disease, 3 mos.; peritoneal fluid, 142 cc.; urine, 330 cc.; blood pressure, initial, 150 mm.; after injection, 155 mm.												
		mM per l.	*gm. per kg.*	*gm. per kg.*		*gm. per gm.*	*m.eq. per kg.*	*m.eq. per kg.*	*m.eq. per kg.*	*mM per kg.*	*mM per kg.*	
Serum. Initial	7.15	15.60	2.61	57.8	3.44	0.9156	102.0	137	3.64	3.20	4.35	
Final	7.34	21.20	2.21	40.0	2.88	0.9385	107.8	142	2.96	2.34	3.35	
Muscle. Initial						0.7747	26.40	41.7	83.4			228
Final						0.7900	34.30	50.1	76.5			287
Peritoneal fluid, per liter			2.21	20.4	3.32		112.0	148				
Urine, excreted							14.9	19.1				
Dog 86; weight 13 kilos; 2200 cc. injected; duration of disease, 3 mos.; peritoneal fluid, 100 cc.; urine, 235 cc.; blood pressure, initial, 130 mm.; after injection, 132 mm.												
Serum. Initial	7.26	17.57	1.11	62.7	2.80	0.9110	111.7	132	4.02	2.72	3.37	
Final	7.37	24.96	0.76	34.0	2.70	0.9482	115.0	127	3.22	1.65	2.42	
Muscle. Initial						0.7735	19.80	32.68				155
Final						0.7899	29.82	44.65				236
Peritoneal fluid, per liter			0.72	11.1			120.0	133				
Urine, excreted							7.2	10.0				

Dog 89; weight 12.8 kilos; 2100 cc. injected; duration of disease, 2 mos.; peritoneal fluid, 270 cc.; urine, 175 cc.; blood pressure, initial, 110 mm.; after injection, 128 mm.

Serum. Initial	7.08	20.00	2.05	68.9		0.9038	106.0	136	7.76			
Final	7.26	30.40	1.61	33.0		0.9470	110.6	141	3.14			
Muscle. Initial						0.7689	23.55	31.40				193
Final						0.7868	29.60	39.10				243
Peritoneal fluid, per liter			1.31	20.7			115.7	148				
Urine, excreted							8.0	11.2				

* *Histological Findings*—The findings apply to sections taken from those regions of the kidney, usually the poles, where some parenchyma was still left. In general, extreme atrophy, flattening and distortion of tissues; marked fibrosis. Acute necrosis of most of glomeruli and cortical tubules; remaining tubules dilated, containing epithelial casts, dense proteins, often concretious masses. Medulla widely scarred with young connective tissue with focal subcapsular scars in cortex. Dog 72 showed also slight ascending infection of some of the large ducts; Dog 86 showed also a focal acute and subacute pyelonephritis; Dog 89 showed a mild acute pyelitis; Dog 95 showed no infection.

the hydronephrotic non-uremic and progressive uremic stages. The calculated results are given in Table IV. The values for P

TABLE IV

Phase Volume Data from All Animals, Grouped for Purposes of Comparison

(F) = gm. of extracellular phase per kilo of muscle
(C) = " " intracellular " " " " "
M = $(F) + (C)$
$\{H_2O\}_C$ = gm. of water per kilo of intracellular phase
σ = standard deviation
P = percentage probability
Δ = differences between means before and after injection
Δ_N = " " " of normal animals and of animals with kidney damage, before injection
Δ_{N_1} = differences between Δ_N and Δ for normal dogs

	No. of Animals	M			(F)			(C)			$\{H_2O\}_C$		
		Mean	σ	P	Mean	σ	P	Mean	σ	P	Mean	σ	P
Normal animals													
		gm.		*per cent*	*gm.*		*per cent*	*gm.*		*per cent*	*gm.*		*per cent*
Before injection...	34	1000			173	±22		827	±22		717	±5	
After injection......	3	1048	±10		204	±11		844	±2		724	±5	
Δ..........		+48	±10	0.00	+36	±12	0.00	+12	±16	42	+4	±8	62
Animals in acute uremia													
Before injection...	5	1000			190	±14		810	±14		716	±2	
Δ_N.........					+17		51	−17		51	−1		100
After injection......	5	1055	±22		227	±18		828	±19		721	±8	
Δ..........		+55	±22	0.00	+37	±15	0.00	+18	±14	34	+5	±4	53
Δ_{N_1}........		+7		76	+1		96	+6		76	+1		91
Animals in chronic non-uremic hydronephrosis													
Before injection...	4	1000			160	±15		840	±15		718	±9	
Δ_N.........					−13		62	+13		62	+1		84
After injection......	4	1066	±14		204	±18		862	±16		725	±9	
Δ..........		+66	±14	0.00	+44	±18	0.00	+22	±16	17	+7	±5	42
Δ_{N_1}........		+18		30	+8		72	+10		66	+3		75

TABLE IV—*Concluded*

Animals in uremic stage of progressive hydronephrosis													
Before injection...	9	1000			201	±29		799	±29		708	±11	
Δ_N.........					+28		44	−28		43	−9		51
After injection......	9	1081	±15		270	±29		809	±39		710	±10	
Δ..........		+81	±15	0.00	+69	±17	0.00	+10	±13	80	+2	±4	62
Δ_{N_1}........		+33		7	+33		12	−2		92	−2		82

are calculated by the method given by Dunn (13), and represent the probabilities, in per cent, that the differences found are due to random sampling. Inspection of Table IV shows that low P values, corresponding to statistically significant differences, are consistently found only when the total muscle volume (*M*) and the extracellular phase (*F*) are compared before and after injection. All other comparisons are of doubtful or of no statistical significance.

It is apparent, from a comparison of the normal dogs with the dogs in acute uremia as a result of having no kidney function for 3 to 5 days, that there is no edema of the skeletal muscle in the uremic animals before the injection. Following the increase in total body water, the increases in the extra- and intracellular phases were not different from those found in dogs with normal kidney function.

It is also apparent that the volumes of the muscle phases in the animals with chronic non-uremic hydronephrosis, both before and after the injections, were the same as those found in the normal animals. The small differences between animals in chronic non-uremic hydronephrosis and normal animals, both before and after the increase in total body water, must be attributed to random sampling. The blood serum and muscle analyses on these hydronephrotic animals were essentially normal. Although approximately 50 per cent of the kidney had been destroyed by increased intraureteral and intrapelvic pressure, the amount of functioning renal tissue remaining was sufficient to maintain normal composition of the internal environment.

It may be concluded, therefore, that dogs with no kidney function, in acute uremia, and dogs in chronic non-uremic hydronephrosis do not store their water in skeletal muscle; and that if additional water is added, it is distributed as in the normal animal.

The statistical data on the animals in the uremic stage of progressive hydronephrosis show that the phase volumes of the original muscle do not differ significantly from those for the muscle of normal dogs. The slight differences found are attributable to sampling. These results indicate that there is no quantitatively significant change in the initial volumes of extra- and intracellular phases of muscle in dogs whose kidney function has been severely impaired.

Following the increase in total body water, the absolute increase in the bulk of 1 kilo of original muscle amounted to 81 gm., which was 33 gm. more than that obtained in the muscle of dogs with normal kidneys. It is probable that this 33 gm. increase is a real difference, for it could occur only seven times in a hundred by sampling, although this probability figure is on the border line of statistical significance. This increase in the bulk of the muscle was entirely accounted for by the increase in the extracellular phase.

In all experiments, the constancy of the percentage of water in the intracellular phase is worthy of special emphasis. This constancy, whether the animals have no kidney function or severely decreased function, suggests that such pathological conditions do not alter the skeletal muscle cell.

The bearing of these findings upon the problem of edema requires discussion. It should be noted first that none of the dogs with acute uremia or with non-uremic hydronephrosis exhibited demonstrable edema, and that of the dogs with progressive uremic hydronephrosis, only one (Dog 95 in Table III) had visible subcutaneous edema which was present at the time the experimental data were obtained. In this dog, no gross deviation in the distribution of fluid between the intra- and extracellular phases of skeletal muscle was found.

It should also be pointed out that edema is not a common concomitant either of acute uremia or of chronic suburemic states associated with severe impairment of renal function. The gross findings in the dogs are therefore in agreement with clinical experience.

It might, however, have been anticipated that in spite of the absence of gross edema there would be a shift in the distribution of fluid in the skeletal muscle in an attempt to store water in the extracellular phases. This turns out not to be the case. The negative findings are nevertheless of positive physiological significance, since they demonstrate the constancy with which the fluid of the muscle is distributed even in the presence of severely impaired renal function.

SUMMARY

1. Reduction in kidney function was produced in dogs by experimental hydronephrosis.
2. The volumes of the extra- and intracellular phases of the skeletal muscle of dogs in acute uremia, chronic non-uremic hydronephrosis, and progressive uremic hydronephrosis, both before and after an increase in total body water produced by the intravenous injection of slightly alkaline isotonic salt solution, were compared statistically with those obtained on normal dogs. The data indicated the following conclusions. (*a*) Dogs with no kidney function, in acute uremia, did not store their water in skeletal muscle; when additional water was added to the body, it was distributed as in normal animals. (*b*) Dogs in chronic non-uremic hydronephrosis, with about 50 per cent of their renal tissue destroyed, maintained the volume of the muscle phases within normal limits. After an increase in total body water, the additional water was distributed as in normal dogs. (*c*) Dogs in progressive uremic hydronephrosis, with the greater part of their renal tissue destroyed, maintained the volume of the muscle phases within normal limits before an increase in total body water. After an increase in total body water, there was some indication that the total bulk of the muscle increased more than in normal animals. This increase was entirely accounted for by the increase in extracellular phase, the intracellular phase remaining constant as in the other experimental animals.
3. There is in these experiments no indication of an influence of diminished kidney function upon the distribution of fluid in skeletal muscle, and consequently no evidence for the occurrence of edema as a result of impaired renal function.

BIBLIOGRAPHY

1. Hastings, A. B., and Eichelberger, L., *J. Biol. Chem.*, **117**, 73 (1937).
2. Eichelberger, L., *J. Urology*, **40**, 366 (1938).
3. Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, **119**, 1 (1937).
4. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
5. Howe, P. E., *J. Biol. Chem.*, **49**, 93 (1921).
6. Robinson, H. W., Price, J. W., and Hogden, C. G., *J. Biol. Chem.*, **120**, 481 (1937).
7. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, **48**, 223 (1921).
8. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, **63**, 461 (1925).
9. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
10. Shohl, A. T., and Bennett, H. B., *J. Biol. Chem.*, **78**, 643 (1928).
11. Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, **93**, 171 (1931).
12. Gersh, I., *Anat. Rec.*, **70**, 311 (1938).
13. Dunn, H. L., *Physiol. Rev.*, **9**, 275 (1929).

PHOSPHOLIPID CHANGES DURING THE PRODUCTION OF FATTY LIVERS IN GEESE

BY EUNICE V. FLOCK, HAROLD R. HESTER,* AND
JESSE L. BOLLMAN

(*From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*)

(Received for publication, December 29, 1938)

When fatty livers were produced in geese by overfeeding, the fat was found to be more saturated than normal, although there was no shift in the usual proportion of liquid and solid fatty acids (4). An investigation has now been made of the quantity and composition of the phospholipids in such livers as shown by the iodine numbers. Best, Channon, and Ridout (1) have shown that, in fatty livers produced in other animals by high fat feeding or by cholesterol feeding, the phosphatide content varies inversely with the total lipid content. Although the percentage of phospholipid in the liver is not changed by a fat diet, considerable variation may be readily produced in the iodine numbers of the phospholipids by varying the type of fat in the diet, as shown by Sinclair (5).

Procedure

A group of geese was overfed with biscuits containing 15 per cent white flour, 76 per cent corn-meal, and 9 per cent corn syrup, seven times every 24 hours, as previously described, for from 8 to 22 days, between December 11 and February 10. Control groups of birds were allowed to eat at will of a mixed diet of 25 per cent corn, 50 per cent wheat, and 25 per cent oats during this period and also in October.

The birds were decapitated, the liver was quickly removed, and samples were taken for the determination of content of glycogen. The remaining liver was ground and samples weighed for total lipid and water content.

---

* Fellow in Comparative Pathology.

Total Lipids—2 to 4 gm. samples were extracted by refluxing for hourly intervals, once with alcohol and then twice with a 3:1 alcohol-ether mixture. Hydroquinone was added as an antioxidant and the solvent evaporated, the final traces being removed in a current of nitrogen. The residue was dissolved in petroleum ether, filtered, dried, and weighed for total lipids. An aliquot was used for cholesterol determination by the method of Liebermann and Burchard as used by Bloor and his coworkers (3).

Fractionation of Lipids—The neutral fat and phospholipids were separated by ice-cold acetone and a small quantity of magnesium chloride, as described by Bloor (2). The phosphorus content of an aliquot of the phospholipid fraction was determined after ashing with sulfuric acid and superoxol by the method of Fiske and Subbarow. Each fraction was saponified and the fatty acids were then freed with excess hydrochloric acid and extracted with petroleum ether. The ether extract was washed with water and the fatty acids were dried and weighed. The fatty acids in each fraction are reported as percentage of the total lipids. The iodine numbers of the fatty acids were determined by the method of Yasuda (6), correction being made for the cholesterol content of the neutral fat fraction.

Results

The total lipid content of the livers of the normal geese studied varied from 5.7 to 10.6 per cent, with a more or less independent variation in liver weight of from 0.99 to 3.74 per cent of the total weight of the goose. Associated with these variations were found changes in the relative proportions of neutral fat and phospholipids and in the iodine numbers of the fatty acids in these. The iodine numbers of the neutral fat were much lower than those of the phospholipids and, indeed, in most cases well below those of the depot fat (Table I). When the neutral fat increased to 60 per cent or more of the total fatty acids, the iodine numbers were definitely decreased, particularly those of the phospholipids.

When the fat content of the liver was increased by stuffing the goose with a high carbohydrate diet, the same kind of changes occurred, although they were much greater on a quantitative basis than those found in the normal goose eating at will (Table I). As the quantity of total lipid in the liver increased, the greatest change was found in the neutral fat fraction. Variations occurred in the cholesterol content but these were independent of the content of total lipid as reported previously. The percentage concentration of phospholipids was greatly decreased, although the total amount expressed as gm. per 100 gm. of total weight

TABLE I

Partition of Liver Fat in Normal Geese and in Geese Subjected to Overfeeding; December to February and October

All weights are expressed per 100 gm. of total weight of goose. Neutral fat is expressed in terms of the fatty acids. Phospholipids are calculated from the P content of the fraction, × 25.

Goose No. and sex	Liver weight	Total lipids		Neutral fat			Phospholipids			Mesenteric fat, I No.
			Weight		Weight	I No.		Weight	I No.*	
Normal geese										
	gm.	*per cent*	*gm.*	*per cent*	*gm.*		*per cent*	*gm.*		
1. F.†	0.99	8.45	0.084	31.2	0.026	72	48.1	0.040	112	80
2. “ †	1.03	7.46	0.077	26.3	0.020	66	54.0	0.042	110	76
3. “	1.05	6.92	0.073	27.2	0.020	62	61.2	0.045	109	62
4. “	1.21	8.43	0.102	43.1	0.044	61	40.0	0.041	106	67
5. M.	1.27	5.74	0.073	29.4	0.021	60	40.4	0.029	102	66
6. “	1.67	5.74	0.096	53.1	0.051	59	27.6	0.026	84	68
7. F.	1.94	5.70	0.110	26.2	0.029	57	43.8	0.048	102	62
8. “	2.35	8.55	0.201	61.6	0.124	54	14.9	0.030	71	70
9. “ †	2.46	8.82	0.217	41.3	0.090	61	34.2	0.074	97	79
10. “ †	2.65	8.65	0.229	39.8	0.091	60	36.9	0.084	101	82
11. “ †	3.00	10.25	0.308	65.4	0.201	55	17.8	0.055	72	87
12. “ †	3.74	10.60	0.396	60.4	0.239	55	19.8	0.078	63	82
Overfeeding										
13. M.	1.81	15.3	0.277	51.4	0.142	54	16.0	0.044	72	60
14. F.	2.00	11.3	0.226	59.5	0.134	53	25.2	0.057	81	58
15. “	2.40	22.4	0.538	73.0	0.393	54	7.5	0.040	72	56
16. “	2.60	13.4	0.348	73.8	0.257	51	17.2	0.060	68	65
17. “	2.60	12.9	0.335	72.2	0.242	56	15.2	0.051	67	62
18. “	2.67	6.2	0.166	50.1	0.083	54	24.2	0.040	72	61
19. “	2.69	12.9	0.347	61.0	0.212	56	14.0	0.048	78	63
20. “	3.50	18.8	0.658	71.7	0.472	50	15.2	0.100	66	65
21.	3.81	26.1	0.994	80.2	0.797	52	10.4	0.103	64	65
22. F.	4.67	25.6	1.196	83.2	0.995	54	8.0	0.096	70	63
23. M.	4.72	18.9	0.892	73.2	0.653	52	9.8	0.087		65
24. F.	4.72	37.6	1.731	89.0	1.540	53	5.2	0.090	68	62

* Iodine number refers to the fatty acids.

† October.

of goose either remained unchanged or showed a small increase. The phospholipids were determined both by the phosphorus content and by the fatty acid content of the acetone-insoluble fat, assuming a composition of 4 per cent phosphorus and 70.5 per cent fatty acids. The values obtained by these two methods agreed closely in the normal livers. In the extremely fatty livers the amount of phospholipid as calculated from the fatty acid content was somewhat higher than from the phosphorus content, owing either to a change in the composition of phospholipid or more likely to incomplete removal of the unusually large amount of neutral fat. The values based on the phosphorus content were considered more reliable and are used in Table I. Some error is introduced thereby in the quantity of neutral fat but it is small compared with the total amount. The iodine numbers of the fatty acids from the neutral fat and phospholipids of the liver as well as of the mesenteric fat of the stuffed geese are in most cases definitely below those found in the control geese. The greatest difference is found in the phospholipids. In the stuffed geese the iodine numbers of the fatty acids from the neutral fat were again lower than those of the mesenteric fat.

It is difficult to determine just what rôle, if any, the phospholipids of the liver play in the production of a fatty liver as a result of a high carbohydrate diet. On the basis of the percentage content of phospholipid in the liver they would appear to have little to do with this process, just as on this same basis they appear to have little to do with the production of a fatty liver as a result of a high fat diet or toxic agents. That they are affected, directly or indirectly, by the production of a fatty liver as a result of a high carbohydrate diet just as by the other methods mentioned is apparent from the marked change which occurs in the iodine numbers of the fatty acids of the phospholipids. The changes found in the liver fat of the goose all occur normally in the goose eating at will but are greatly accentuated by the stuffing process. The constant disparity in the degree of saturation of the fat in the liver and in the depots in the goose which is making large quantities of fat from carbohydrate suggests that the liver is taking an active part in the process and is not merely being passively flooded with fat from the depots.

SUMMARY

When a rapid synthesis of fat from carbohydrate is produced in the goose by forced feeding of a high carbohydrate diet, the great increase in total lipids of the liver is found to be due chiefly to an increased amount of neutral fat.

The phospholipids of the liver are greatly decreased in terms of per cent of total lipid but in terms of total amount per 100 gm. of goose they are unchanged or slightly increased. Decreases in the iodine numbers of the fatty acids of the lipids studied occur in the following order: phospholipids, neutral fat of the liver, and mesenteric fat.

BIBLIOGRAPHY

1. Best, C. H., Channon, H. J., and Ridout, J. H., *J. Physiol.*, **81**, 409 (1934).
2. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
3. Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, **52**, 191 (1922).
4. Flock, E., Bollman, J. L., Hester, H. R., and Mann, F. C., *J. Biol. Chem.*, **121**, 117 (1937).
5. Sinclair, R. G., *Physiol. Rev.*, **14**, 351 (1934).
6. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).

A LOW PHOSPHORUS DIET AND THE RESPONSE OF RATS TO VITAMIN D_2*

BY H. SCHNEIDER AND H. STEENBOCK

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, January 14, 1939)

For the past decade or more the experimental production of rickets in rats has generally involved the use of cereal rachitogenic diets (Sherman and Pappenheimer (1), McCollum *et al.* (2), and Steenbock and Black (3)). Metabolic studies have revealed that the rickets thus produced is referable to a phosphorus deficiency in the growing animal, as indicated by a lowered inorganic P content of the blood and a lowered ash content of the bones. However, Bruce and Callow (4) and Lowe and Steenbock (5) have shown that the cereal, so called "low phosphorus" diets are so in effect only because of the low availability of their phytin phosphorus. Giri (6) reports that 50 to 70 per cent of the phosphorus of cereals is phytin phosphorus. Maize, the main constituent of the Steenbock-Black diet, is reported as having 69.1 per cent of its P present as phytin P. Giri (6) has also shown that the phytase activity of cereals is in inverse relation to the phytin content. Patwardhan (7) has demonstrated a low phytase activity in the intestine of the rat. Phytases evidently can introduce a factor of uncertainty in studies in phosphorus metabolism, for it is obviously essential that all sources of physiologically available phosphorus be known and controlled. Synthetic diets actually low in total phosphorus would offer obvious advantages over those composed of naturally occurring foods.

---

* A preliminary report of this investigation was presented at the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, April 2, 1938.

Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Basal Diet—In designing a low P diet otherwise nutritionally adequate the Ca content was fixed at a level commonly met with in synthetic diets; *viz.*, 0.57 per cent. The vitamin D content was varied. Suitable proteins were available only from limited sources. Most proteins are either biologically incomplete and too high in P or too difficult of preparation in quantity. Jones (8) has recently suggested the use of alcohol-extracted blood fibrin in rachitogenic diets. Commercial egg white (Stein-Hall) was finally selected as the most suitable. Before use it was steamed for 3 hours to destroy toxic factors (Parsons (9)). The water-soluble vitamins were at first introduced in the form of water extracts of yeast in which the P content had been reduced by precipitation with iron salts. Supplements of alfalfa extracts were also used. Later these were replaced with vitab,[1] a concentrated extract of rice bran and whey. Salts were provided as Wesson salts (10) from which the P-containing salts had been eliminated by substitution with KCl, Ca lactate, and Fe citrate.

The selection of the other components of the diet offered no particular problem, inasmuch as they were available in quantity and relatively pure. For most of the carbohydrate a commercial glucose of high purity (cerelose) was selected. It had a P content less than 0.01 per cent. For fat a commercial refined cottonseed oil (Wesson oil) was chosen. Vitamin A was supplied as the provitamin, β-carotene, 120 micrograms per rat per week, dissolved in Wesson oil. When vitamin D was added, it was supplied as a Wesson oil solution of calciferol. The Wesson oil itself was found to contain less than 0.001 per cent P.

For convenience the vitab was incorporated in the diet by evaporating an appropriate amount of a water solution on cooked starch at room temperature before a fan. When so prepared, no loss of growth-promoting properties was observed after a storage period of 3 months.

The basal diet (Diet R-14) had the following composition: cerelose (glucose) 49, egg white 18, cooked starch 20, vitab 4, P-free salts 4, cottonseed oil 5. It contained 0.04 per cent P. When supplemented with 1.80 parts of $NaH_2PO_4 \cdot H_2O$ (Diet NR-14), it contained 0.41 per cent P. It was then identical in

---

[1] **Vitab, Type II, was kindly supplied by Vitab Products, Inc., Emeryville, California.**

P content with Diet R-14 when supplemented with 4 per cent of Wesson salts (Diet WR-14). That the organic constituents were satisfactory for normal nutrition was demonstrated with Diet WR-14 supplemented with 600 I. U. of vitamin D per rat per week. On it growth was entirely normal; male rats gained on an average 4.0 gm. daily in 6 weeks. After 7 weeks, when two males and two females reared on the diet from a weanling weight of approximately 50 gm. each were mated, pregnancy was induced and normal young were born at term. The does nursed the young in litters of six. After weaning at 4 weeks, a second generation was also reared. The animals of this litter grew at approximately the same rate as the first. A male and a female of this second generation were bred and a third generation litter was born, suckled, and weaned.

Effect of Low Phosphorus Diets without Vitamin D

Growth—When young growing rats weighing 50 to 60 gm. were placed on the low P diet (R-14), they grew at a much slower rate than rats on the same diet supplemented with adequate amounts of P (Diet NR-14) (Fig. 1). Growth on the low P diet reached its maximum at about the 4th week, followed by a rapid decline in weight and death. Most of the animals died by the 6th week. Only one animal out of 50 survived for 8 weeks.

Rachitogenesis—Diet R-14 was strongly rachitogenic. After 12 days the distal ends of the radii and ulnæ revealed a rachitic metaphysis as wide as that produced in 21 days by a cereal rachitogenic diet (Ration 2965, Steenbock-Black). By the 4th week skeletal abnormalities were marked. Muscular tone was poor and a general lethargic condition prevailed. In advanced cases the hind limbs were paralyzed. Loss in weight occurred in spite of good food consumption. At autopsy, ventral spinal curvature, single or double, was easily recognized in the lumbar region. Sternal curvature also occurred. The costochondral junctions were enlarged. All bones were soft.

Other Symptoms—In the 3rd or 4th week an encrustation of dried blood and exudate accumulated around the nares. Breathing was labored and râles were audible. These symptoms were not observed in rats kept under identical conditions except for adequate supplements of P. Eppright and Smith (11) have reported the production of nasal encrustations on diets low in mineral elements. They found the symptoms to disappear after dietary additions of calcium phosphate. These authors concluded that the respiratory difficulties were not referable to pulmonary infections, as postmortem examination failed to reveal any pulmonary lesions. In our own experiments the locus of the exudates was likewise confined to the nasal pharyngeal region.

Although an adequate supply of β-carotene was furnished in the diet, the possibility that a diet deficient in phosphorus might influence unfavorably its absorption or transformation to vitamin A was investigated. Two rats exhibiting marked signs of P deprivation after 4 weeks were killed and the vitamin A content of the livers determined by a modification of the method of Davies (12). A vitamin A content of 75 and 108 blue units respectively was found. A deficiency of this dietary essential was therefore excluded.

Hematopoiesis—Orten, Smith, and Mendel (13) have reported that salt-deficient diets produced a polycythemic anemia which yielded to dietary supplements of $CaCO_3$ and, less effectively, to $FeCl_3$. As their low salt ration contained casein as a source of P, there was a possibility that this P might have been responsible for the polycythemia. This was given some credence from the work of Swanson, Timson, and Frazier (14) who reported less drastic changes in the blood when edestin was fed in place of casein.

Determinations of the hemoglobin content of tail blood in rats on our low P ration, Diet R-14, made at weekly intervals by the method of Newcomer (15) failed to reveal that lack of P had any effect on hematopoietic activity. Four rats on the low P diet had an average Hb value of 14.65 gm. per 100 cc. at the end of the 4th week. Two of these died during the 5th week. The initial Hb value of the four rats was 13.15 gm.

Creatine Metabolism—It has already been remarked that the loss in weight after the 4th week on Diet R-14 was not due to an inanition. It has also been remarked that these rats on the low P ration were extremely weak. Since P is indispensable for the normal metabolism of muscle, it appeared desirable to determine the creatine excretion. Two rats were placed on Diet R-14 for 2 weeks in a metabolism cage for collection of urine and feces.

After the 14th day, collections of urine under toluene were made in 3 day periods. Creatinine was determined according to Shaffer (16) and creatine according to the method of Folin (17). Collections were made until the death of the animals.

Throughout the experiment creatinine excretion remained constant when referred to body weight, an average of 1.27 mg. of creatinine N being excreted per 100 gm. per day. This constant excretion was observed both during growth and the eventual loss in weight as the animal declined. However, creatine excretion was observed to be fairly constant only during the growth of the rats and rose sharply when loss of weight began. The creatine excretion during the period of weight loss rose to 1.11 mg. per rat per day from a previous level of 0.08 mg. per rat per day. It is an open question whether this outpouring of creatine may be attributed directly to some aberration in the metabolism of creatine-phosphate or whether it is merely referable to a general tissue disintegration.

Phosphorus Content of Tissues—P was determined in various tissues of rats on Diet R-14. It is well known that low P diets of the cereal type result in a lowered blood P and a diminished P content of bone, but, in general, soft tissues have been found to maintain a constant P content. Thus Hentschel and Zoeller (18) found no difference in the total P content of muscle of normal rats and rats on the rachitogenic diets of McCollum, Sherman and Pappenheimer, and Steenbock and Black.

Three diets were fed; *viz.*, a synthetic low P diet (R-14), Diet R-14 with its P content increased to adequate levels with NaH_2PO_4 (Diet NR-14), and a cereal "low P" diet (Ration 2965). 50 to 60 gm. weanling rats from our stock colony were placed on these diets and at suitable intervals several rats were killed in each group after they were etherized and blood withdrawn with a syringe from the abdominal aorta. Inorganic P and total Ca were determined in the sera. The liver, heart, brain, the left femur, and the right gastrocnemius were dissected out, dried at 102° for 48 hours and analyzed for total P by ashing under $Mg(NO_3)_2$ in a muffle furnace by a modified Fiske-Subbarow method (19).

From the resultant data (Table I) it is apparent that blood and bone were the only tissues which showed any decrease in phosphorus content. Even after 45 days, when the rats of Diet R-14 were in extremis, liver, heart, and brain showed no significant alteration in P content. Although a low level of 3.01 mg. of P per 100 cc. of serum was reached after 14 days, and occasionally a level as low as 1.35 per 100 cc. after 21 days, after 14 days the P sometimes increased as the rats began to lose weight. It is also interesting to note the occurrence of a hypercalcemia on Diet R-14 similar to that observed on the cereal rachitogenic diet, Ration 2965. This is notable, since the Ca content of Diet R-14 was at the "normal" level of 0.57 per cent as compared with the surfeit of Ca, 1.2 per cent supplied by Ration 2965.

TABLE I

Tissue Analyses of Rats on Diets of Varying Phosphorus Content; Averages for Three Rats

Group and diet	Days on experimental diet	Mg. total P per gm., dry weight					Mg. per 100 cc. Blood serum	
		Liver	Bone	Heart	Muscle	Brain	P	Ca
Group 0. Stock rats, 50-60 gm.	0	9.65	77.0	13.0	11.6	16.6	10.6	11.9
Group 1. Low P diet, R-14	14	11.0	50.0	11.8	10.8	16.3	3.01	13.4
	35	11.1	40.5	11.6	10.5	15.7	5.78	15.9
	45	11.3	39.2	11.8	10.3	15.9	4.20	12.5
Group 2. Diet R-14 $+NaH_2PO_4$	14	11.5	82.9	11.7	10.4	15.6	8.90	9.8
	35	11.8	87.6	10.9	10.1	15.9	8.72	10.2
	45	11.6	88.8	10.8	10.2	16.2	7.64	9.4
Group 3. Ration 2965	14	10.6	61.5	11.6	10.6	15.3	3.53	12.1
	35	11.6	41.1	11.6	10.1	16.5	6.07	12.6
	45	11.4	37.8	11.8	9.7	16.8	4.57	11.3

A diet of adequate P content (Diet NR-14) was observed to maintain the high serum inorganic P, as observed in rats on a stock diet by Bethke, Steenbock, and Nelson (20).

Effect of Low Phosphorus Diets with Vitamin D

Therapeutic Effects—Thirty weanling rats, weighing 50 to 60 gm., were divided into three groups of ten each. Group 1 was fed Ration 2965 for 3 weeks; Group 2, Diet R-14 for 3 weeks; Group 3, Diet R-14 for 2 weeks. At the end of these periods the animals were fed 3.3 I. U. of vitamin D as crystalline calciferol dissolved in Wesson oil in 8 days according to the U. S. P. XI technique. On the 10th day the rats were killed and the distal ends of the radii and ulnæ sectioned and stained with silver nitrate. It was found that rats made rachitic on Diet R-14 showed approximately the same response to vitamin D as those given the cereal rachitogenic diet, Ration 2965, as indicated by the line test. But the rats on Diet R-14 did not increase in body weight during the period of supplementation, and in fact lost on an average of 1.8 gm. Rats on Ration 2965 averaged a gain of 6.6 gm. It is recognized that healing may occur spontaneously if the animal fasts. In this instance the animals did not fast, but as rats on Diet R-14 suffer a rapid loss in weight beginning in the 4th week, no particular significance was attributed to the loss in weight when first observed.

Prophylactic Effects—The early administration of vitamin D prevented the incidence of rickets on Diet R-14. Skeletal abnormalities did not appear, enlarged costochondral junctions were not observed, and examination of sections of the wrists by staining with silver nitrate failed to reveal any sign of rickets. The nasal discharge which was characteristic of rats on Diet R-14 without vitamin D did not appear. But what was most striking were the growth effects. Without vitamin D, it will be recalled, growth on Diet R-14 was subnormal but none the less definite, a gain of 35 gm. during a 3 week rachitogenic period being common. However, when 1200 I. U. per week of vitamin D as calciferol were fed, a loss in weight occurred (Fig. 1), and in 3 weeks the rats had not regained their initial weight. Similar results were obtained with smaller amounts of vitamin D (Fig. 1), although the loss in weight was less. Ultimately growth was resumed, but the rate was approximately one-third of that shown by rats which were developing rickets on the same diet in the absence of vitamin D. The same inhibitory action of the vitamin was demonstrated when vitamin D was added to Diet R-14 after varying periods of rachitogenesis.

To our knowledge there is no previous account of vitamin D causing a loss in weight or acting as an inhibitor of growth. Thus Shohl *et al.* (21) in studying the effects of a "low P" diet used Ration 2965 and supplemented it with 2 per cent cod liver oil.

The growth curves he reported gave no indication of any inhibition. Similarly, in our own laboratory when Ration 2965 was supplemented with 1200 I. U. of vitamin D as calciferol per rat per week, there was no indication of such an effect. The inhib-

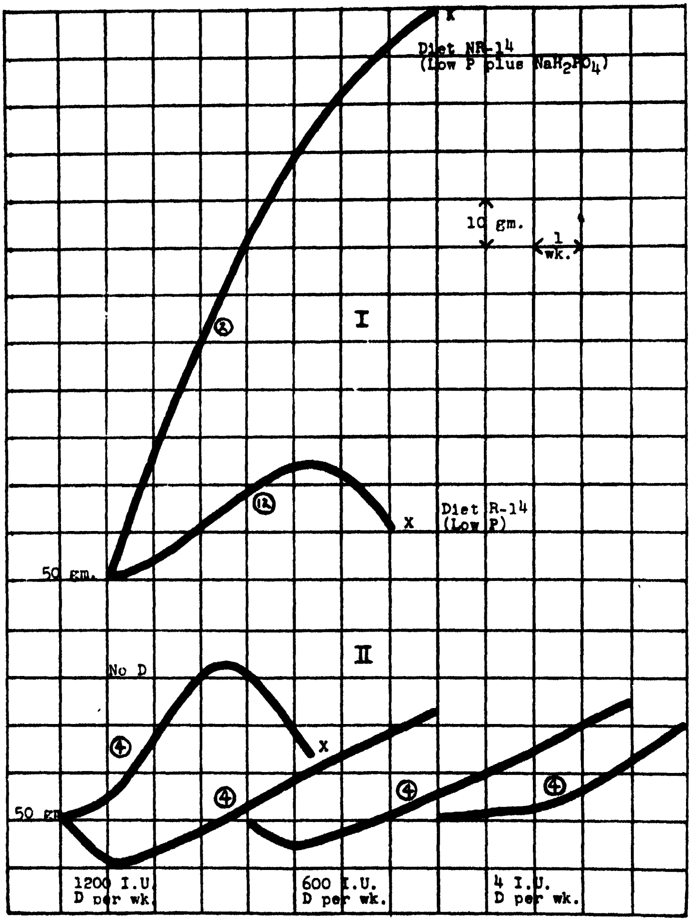


FIG. 1. Growth curves of rats on (Part I) low phosphorus and adequate phosphorus diets, without vitamin D; and (Part II) on low phosphorus diet (R-14) with various amounts of vitamin D. The figures in circles represent the number of rats; *X*, died; *K*, killed.

itory influence of the vitamin could not be attributed to toxicity, since the same amount of vitamin D when fed as a supplement to Ration 2965 was without effect, and the same type of inhibition was obtained on Diet R-14 when amounts as little as 4 I. U. per rat per week were fed (Fig. 1). By supplementation with vitamin D, rats were maintained, at the inhibited growth rate, for as long as 20 weeks. Pathological changes observable after such long periods of maintenance will be reported in a subsequent paper (22).

Ca and P Retention, Growth and P Content of Tissues—Twelve rats weighing from 50 to 60 gm. were fed the following diets: Group 1, Ration 2965; Group 2, Ration 2965 plus vitamin D; Group 3, Diet R-14; Group 4, Diet R-14 plus vitamin D. During the entire experiment food consumption was controlled daily by the paired feeding method. It was soon evident that the rats on the supplemented diets would be the pace-makers; especially marked was the diminished and slow consumption of Diet R-14. On the cereal rachitogenic diet the tendency to restrict consumption with the addition of supplements was less evident. After 3 days on the diets without vitamin D the excreta were collected for 1 week; vitamin D additions were then made, with collection of excreta in a similar manner during the 2nd and 3rd weeks. Groups 2 and 4 received 50 I. U. per day as calciferol in Wesson oil, by dropper. The combined urine and fecal collections were dried and ashed for Ca determinations by the oxalate-permanganate method; P determinations were made by a modified Fiske-Subbarow procedure. At the conclusion of the feeding trials the animals were killed and tissues analyzed as described before. The growth record and Ca and P balances are presented in Table II, tissue and blood serum analyses in Table III.

From Table II it is apparent that on Diet R-14, as on Ration 2965, administration of vitamin D resulted in an improved retention of Ca and P. Particular attention is called to Groups 3 and 4. In the 3rd week the rats in Group 3 were continuing in negative balance of these elements, although they were increasing in weight. When vitamin D was added as in Group 4, the balance became positive; yet the rats gained only slightly in weight. If as in Group 3 an animal is in negative P balance and at the same time is growing, then the P used for growth must have come from tissues other than those responsible for the growth increments. Analyses indicated that blood and bone were the tissues thus drawn upon. However, when vitamin D was given, although Ca and P retention were improved, growth was halted, and P went to blood and bone to restore them to their normal

TABLE II

Ca and P Balances of Rats on Low P Diets; Averages of Three Rats, Paired Feeding

Group No.	Wk.	Gain in weight	Intake		Retention		Remarks
			Ca	P	Ca	P	
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	
1	1st	13.3	626	141	85	42.7	Ration 2965; no vitamin D
	2nd	5.7	557	126	−0.3	23.3	" 2965; " " "
	3rd	4.7	475	107	32.3	18.9	" 2965; " " "
2	1st	14.6	Same as		82	38.7	" 2965; " " "
	2nd	5.0	Group 1		40.7	39.1	" 2965 + " "
	3rd	4.7			95.6	39.5	" 2965 + " "
3	1st	12.0	172	20	−4.4	7.9	Diet R-14; no " "
	2nd	4.3	142	16	−40.5	3.8	" R-14; " " "
	3rd	3.3	145	16	−17.9	−1.9	" R-14; " " "
4	1st	10.4	Same as		−4.7	9.0	" R-14; " " "
	2nd	−0.7	Group 3		−10.5	6.9	" R-14 + " "
	3rd	1.3			17.6	10.9	" R-14 + " "

TABLE III

Tissue Analyses of Rats on Low P Diets, with and without Vitamin D_2; Averages for Three Rats

Group and diet	Vitamin D	Mg. total P per gm., dry weight						Mg. per cent	
								Blood serum	
		Liver	Bone	Heart	Muscle	Brain	Testes	P	Ca
Group 1. Ration 2965	None	11.3	44.1	13.0	11.4	16.8	16.1	4.1	10.3
Group 2. Ration 2965	50 I. U. per day, for 2 wks.	10.2	64.5	13.2	12.4	16.5	16.2	5.5	12.4
Group 3. Diet R-14	None	11.3	36.9	13.9	12.0	16.2	16.3	2.2	9.5
Group 4. Diet R-14	50 I. U. per day for 2 wks.	11.2	44.5	14.5	12.5	16.3	16.7	5.2	12.6

phosphorus levels (Table III). While P was being diverted to blood and bone, growth became static, for, as already apparent from our analyses, the P content of the soft tissues tends to remain constant.

Since most theories of the mode of action of vitamin D have been evolved from experimental data accumulated with the use of Ration 2965, the question arises, how do the findings here presented fit in with current theories? Bills (23) in his review points out that the view was once held that the action of vitamin D was confined to the gut. Nicolaysen (24) revived this older theory when he found that in isolated loops of the intestine the administration of vitamin D increased the rate of absorption of Ca salts, but that no effect was observed when phosphates were used. Nicolaysen arrives at the conclusion that the primary effect of vitamin D is to increase the rate of Ca absorption from the gut. In view of the plethora of Ca in the gut and blood it is difficult to attribute the calcifying effects of vitamin D to an increase in the absorption of what already must be furnished in adequate amounts. Certainly any theory of the mode of action of vitamin D in rachitogenic diets must consider and include effects on P as well.

A distinction should be made between the demands of bone and the soft tissues for phosphorus. Nicolaysen (25) has calculated the weekly P requirement of a rat growing 1 gm. per day as 31.5 mg. Assuming that the P requirements are proportionate to the amounts retained by the various tissues, since approximately 20 per cent of the P of the body is found in the soft tissues, 6.3 mg. of P could be accepted as the weekly requirement for soft tissue development and 25.2 mg. of P as the weekly bone requirement. It is obvious from Table II that supplementing Ration 2965 with vitamin D resulted in a retention of P ample for the requirements of both soft and bone tissue. This shows why a rachitogenic diet composed of cereals cannot be used to demonstrate the diversion of P to bone by a supplement of vitamin D.

From examination of the relation of the blood changes to these differences in growth it is apparent that without vitamin D the inorganic P content of the blood was low (2.2 mg. per cent), but growth proceeded; with vitamin D the inorganic P content of the blood was raised (5.20 mg. per cent), but growth was halted.

As the soft tissues were seeking P, and the P supply in the blood was increased, it follows that either the increase in inorganic P in the blood represented a form of P not available for growth, or that the growth of the tissues was inhibited by the presence of vitamin D directly. The latter is improbable, since adequate P in the diet gave normal growth in the presence of identical amounts of vitamin D.

The hypothesis that the increased inorganic P in the blood is not simple inorganic P has been suggested. Fractionation of the forms of Ca and P in the blood by Benjamin and Hess (26) and Benjamin (27) indicate that a diffusible, adsorbable, Ca-P complex provides the substance for bone calcification. Vitamin D has been shown to increase the amount of this adsorbable P.

SUMMARY

A synthetic diet low in phosphorus and free of vitamin D but adequate in other nutritional requirements of the rat has been devised. When supplemented with sufficient phosphorus and vitamin D, it sufficed as the sole source of nutrients for three successive generations of rats. Without these supplements it produced rickets in from 12 to 14 days and brought on severe skeletal deformities in 6 weeks. Growth stopped after 4 to 5 weeks, followed by a rapid decline in weight and death by the 6th week. Phosphorus analyses of tissues and data on creatine and creatinine excretion did not provide the explanation.

When vitamin D was added to the low P diet, the resultant metaphyseal healing was identical with that observed when like amounts were fed in cereal diets. However, when the low P diet was supplemented with vitamin D, a cessation or inhibition of growth occurred which did not result under the same circumstances with cereal rachitogenic diets. Calcium and phosphorus balances and tissue analyses revealed that vitamin D induced the utilization of P by bone, thereby depriving the soft tissues of their supply of P, which in turn inhibited growth.

BIBLIOGRAPHY

1. Sherman, H. C., and Pappenheimer, A. M., *J. Exp. Med.*, **34**, 189 (1921).
2. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, **47**, 507 (1921).

3. Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).
4. Bruce, H. M., and Callow, R. K., *Biochem. J.*, **28**, 517 (1934).
5. Lowe, J. T., and Steenbock, H., *Biochem. J.*, **30**, 1126 (1936).
6. Giri, K. V., *Indian J. Med. Research*, **25**, 869 (1938).
7. Patwardhan, V. N., *Biochem. J.*, **31**, 560 (1937).
8. Jones, J. H., *J. Nutrition*, **15**, 269 (1938).
9. Parsons, H. T., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **97**, p. xxx (1932).
10. Wesson, L. G., *Science*, **75**, 339 (1932).
11. Eppright, E. S., and Smith, A. H., *J. Nutrition*, **14**, 21 (1937).
12. Davies, A. W., *Biochem. J.*, **27**, 1770 (1933).
13. Orten, J. M., Smith, A. H., and Mendel, L. B., *J. Nutrition*, **12**, 373 (1936).
14. Swanson, P. P., Timson, G. H., and Frazier, E., *J. Biol. Chem.*, **109**, 729 (1935).
15. Newcomer, H. S., *J. Biol. Chem.*, **55**, 569 (1923).
16. Shaffer, P. A., *J. Biol. Chem.*, **18**, 525 (1914).
17. Folin, O., *J. Biol. Chem.*, **17**, 469 (1914).
18. Hentschel, H., and Zoeller, E. Z., *Z. Kinderheilk.*, **44**, 146 (1927).
19. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
20. Bethke, R. M., Steenbock, H., and Nelson, M. T., *J. Biol. Chem.*, **58**, 71 (1923–24).
21. Shohl, A. T., Brown, H. B., Chapman, E. E., Rose, C. S., and Saurwein, E. M., *J. Nutrition*, **6**, 271 (1933).
22. Schneider, H., and Steenbock, H., *Wisconsin Agric. Exp. Stat., Bull. 442*, 77 (1938).
23. Bills, C. E., *Physiol. Rev.*, **15**, 1 (1935).
24. Nicolaysen, R., *Biochem. J.*, **31**, 323 (1937).
25. Nicolaysen, R., *Biochem. J.*, **31**, 105 (1937).
26. Benjamin, H. R., and Hess, A. F., *J. Biol. Chem.*, **100**, 27 (1933).
27. Benjamin, H. R., *J. Biol. Chem.*, **100**, 57 (1933).

THE CONVERSION OF PROTEIN TO GLUCOSE IN DEPANCREATIZED AND PHLORHIZINIZED DOGS

By J. S. GRAY, A. C. IVY, and F. P. CUTHBERT

(From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago)

(Received for publication, January 11, 1939)

The phlorhizinized dog has been used extensively for the quantitative estimation of glucose formation from protein, amino acids, other organic acids, and products of metabolism. The depancreatized dog has not been similarly used; *i.e.*, fasted and then fed a pure protein. This work was undertaken to determine whether the depancreatized dog converts protein to glucose to the same extent as the phlorhizinized dog when the experimental conditions are as analogous as possible. For this purpose the glucose yield of casein was ascertained by similar procedures in both depancreatized and phlorhizinized dogs. And, since the yields in the depancreatized animals might be influenced by the unavoidable removal of pancreatic acinous function, the glucose yield of casein was also determined in phlorhizinized dogs after ligation of the pancreatic ducts.

Methods

Phlorhizinized Dogs. Intact—On the 3rd day of fasting and on each day thereafter normal dogs were injected subcutaneously with 1 gm. of phlorhizin (Merck) suspended in 10 cc. of olive oil, according to the method of Coolen (1895). It was found necessary to recrystallize the phlorhizin by the method of Deuel and Chambers (1925). The glucose to nitrogen ratio had usually reached a constant level by the 4th day on phlorhizin, at which time from 20 to 30 gm. of casein (Merck's according to Hammarsten) were given by stomach tube. One series of these animals was fed 100 gm. quantities of a special bread (soy bean) with a high protein content. The animals were used for a daily determination only for as long as they remained in good condition and their urine remained free of albumin.

Ligated Pancreatic Ducts—The same procedure was employed, except that the dogs were used 2 or 3 weeks after the pancreatic ducts had been ligated and the pancreas separated from the duodenum. They were all in excellent condition at the time the experiment was started. At the conclusion of the experiment autopsy examinations of the animals were made in order to establish that the pancreatic ducts had not regenerated. These animals did not receive raw pancreas in their diet. A series of these animals was also fed the special bread.

Depancreatized Dogs—Normal dogs were depancreatized, then standardized for several weeks on insulin and a diet including raw pancreas. At the beginning of an experiment they were deprived of food and insulin. On each day of fasting they were given 500 cc. of 0.9 per cent sodium chloride under the skin in order to combat the dehydration to which they are subject. Preliminary experiments had convinced us that this precaution is essential in order to maintain the animals in good condition and to insure a good yield of "extra sugar." Usually by the 3rd day of fasting without insulin the glucose to nitrogen ratio had reached a constant level, and the animals were fed casein or the special bread. Ordinarily these dogs were used for determinations on only 2 or 3 successive days, since they fail to recover from longer experimental periods.

Urine was collected under toluene from metabolism cages over 24 hour periods, each period being marked by catheterization. Urinary sugar was determined by the Benedict method (1911). Urinary nitrogen was determined by the Koch and McMeekin (1924) micromethod, the nitrogen content of the casein and bread by the macro-Kjeldahl method. The extra glucose was calculated by the Lusk method.

Results

Glucose to Nitrogen Ratio—It has been suggested that no difference exists between the glucose to nitrogen ratio of the fasting depancreatized and the fasting phlorhizinized dog (Dann, Chambers, and Lusk, 1931–32; Dann, 1933; Cori and Cori, 1935). Since the interpretation of our results depends on whether such a difference exists, it seemed necessary to investigate this matter, although the suggestion was based on the results obtained in only two thyroidectomized animals.

It was found that the average glucose to nitrogen ratio of forty-two fasting dogs on phlorhizin for 4 days was 3.55 ± 0.06 (maximum 4.38, minimum 2.26). The average glucose to nitrogen ratio of thirty-two fasting depancreatized dogs without insulin for 3 days was 2.91 ± 0.09 (maximum 4.37, minimum 1.31). The average glucose to nitrogen ratio of nine fasting phlorhizinized dogs with ligated ducts was 3.60 ± 0.08 (maximum 4.07, minimum 2.95). In order to avoid the necessity of making a subjective selection of the "typical" fasting ratio for each animal, the fasting ratios of only the 4th day for the phlorhizinized dogs and of only the 3rd day for the depancreatized dogs were considered in arriving at these average values. As a result the range of variation is perhaps wider than it might otherwise be. However, similar variations are to be found in the literature.

Ligation of the pancreatic ducts did not alter the glucose to nitrogen ratio of phlorhizinized dogs. It is clear, however, that an experimental difference between the glucose to nitrogen ratio of the fasting phlorhizinized and depancreatized dogs does exist. In accordance with many previous observers, we occasionally obtained ratios of approximately 2.80 in phlorhizinized animals; but unselected data recorded in the literature show that of 100 phlorhizinized dogs only 15 per cent had ratios below 3.0.

During the course of these experiments it was found incidentally in a series of seven dogs which were sacrificed after the daily administration of phlorhizin for from 16 to 26 days that the liver was devoid of gross and microscopic fat (Sudan III). On a shorter period of phlorhizin administration we found, as is well known, that the liver was fatty.

Glucose Yield of Casein—The results are shown in Tables I to III. In Table I, the results on the duct-ligated animals are given in some detail, since data on such animals are not available in the literature. In Table II the averaged results of the glucose yield of casein in the three types of animals are given; the analyses have been averaged and presented in the form of a single determination. In Table III the glucose yields of casein are compared with those of the special high protein bread.

The intact phlorhizinized dog yielded an average of 48 per cent glucose from casein, which confirms Janney's (1915) value of 48 per cent. The depancreatized dog yielded an average of 43.5 per cent glucose from casein. This determination has not been made before in the depancreatized dog by the method of urinary analysis. The results of Ralli, Canzanelli, and Rapport (1931), who determined the R.Q. of the depancreatized dog fed casein,

TABLE I

Response of Duct-Ligated Phlorhizinized Dogs to Casein

Dog No.	Glucose		Nitrogen		Glucose to nitrogen ratio		Extra glucose		N fed
	Fasting	Casein*	Fasting	Casein*	Fasting	Casein*		Yield	
	gm.	*gm.*	*gm.*	*gm.*			*gm.*	*per cent*	*gm.*
1	15.90	20.77	4.57	6.32	3.49	3.25	11.07	48.5	3.55
2	30.05	24.65	8.45	8.30	3.57	2.97	7.69	33.9	3.55
3	21.00	19.62	6.12	7.22	3.44	2.72	7.00	30.9	3.55
4	12.55	15.40	3.98	5.68	3.15	2.70	7.35	38.2	3.55
5	20.09	17.72	7.12	6.91	2.95	2.58	7.21	34.3	3.44
Average....	19.92	19.63	6.05	6.88	3.32	2.84		37.1	

* Average of two tests. The amount of pure casein fed was calculated from the nitrogen content, the factor 6.41 (Janney, 1915) being used.

TABLE II

Averaged Results with Casein*

Preparation of dog	No. of dogs used	No. of tests	Casein fed	N fed	Urine sugar	Urine N	Glucose to nitrogen ratio	Glucose yield
			gm.	*gm.*	*gm.*	*gm.*		*per cent*
Intact, phlorhizinized	6	11	Fasting		24.35	7.13	3.41	
			30.12	4.72	28.07	8.63	3.24	48.6
Duct-ligated, phlorhizinized	5	10	Fasting		20.09	6.05	3.32	
			22.21	3.53	19.63	6.88	2.84	37.1
Depancreatized	4	6	Fasting		11.24	3.90	2.88	
			31.4	4.92	16.79	5.98	2.81	43.5

* The results have been averaged and presented in the form of a single determination for each type of animal.

implied a yield of approximately 50 per cent. However, their animals received 50 gm. of raw pancreas with the casein. The duct-ligated phlorhizinized dog yielded only 37 per cent glucose from casein. It is to be noted in Table II that on feeding casein the glucose to nitrogen ratio definitely decreased in the duct-ligated dogs, but not in the depancreatized dogs. The glucose yields when the more complex food, a special high protein bread, was fed confirm the differences noted for casein in the different canine preparations, except that the yield in the duct-ligated dogs is not significantly different from that in the depancreatized animals.

TABLE III

*Glucose Yields of Dogs Fed Casein and Bread**

The values are given in per cent.

Dog No.	Intact, phlorhizinized		Duct-ligated, phlorhizinized		Depancreatized	
	Casein	Bread	Casein	Bread	Casein	Bread
1	44.7	28.0	48.5	22.8	47.0	21.0
2	51.3	26.8	33.9	21.7	43.8	23.2
3	31.9	29.1	30.9	25.6	38.6	23.8
4	48.5	29.4	38.2	19.5	44.0	
5	52.9	30.5	34.3	16.6		
6	55.9	28.7		20.8		
Average........	47.5	28.7	37.1	21.1	43.3	22.7
	11†	21†	10†	30†	6†	8†

* This special bread contained 22.8 per cent protein, 20.5 per cent of digestible carbohydrate by chemical analysis, and 3.65 per cent nitrogen.

† These numbers refer to the total number of individual tests.

Recovery of Ingested Glucose—As an aid in the interpretation of the observed results, it is desirable to know how much ingested glucose may be recovered in the urine of the phlorhizinized and depancreatized dogs. Barker, Chambers, and Dann (1937) have recently obtained an average recovery of 95 per cent in fasting depancreatized dogs in twenty-one experiments. We have fed 10 and 15 gm. of glucose to one fasting depancreatized dog and recovered 93 per cent in one instance and 87 per cent in the other. These values are within the range reported by the investigators just mentioned and also by Minkowski (1893), Allard (1908), and Moorhouse and collaborators (1915), who fed glucose in addition to a meat diet.

In regard to the recovery of glucose in the fasting phlorhizinized dog, the values reported in the literature vary from 46 to 108 per cent. The average of all the values (56) in the literature, including two of our own (82 per cent), is 87 per cent (Ringer, 1912; Ringer and Frankel, 1914; Csonka, 1915; Sansum and Woodyatt, 1916; Olmsted, 1920; Ringer, 1923–24; Nash and Benedict, 1923; Nash, 1925; Deuel and Chambers, 1925; Wierzuchowski, 1927; Deuel, Wilson, and Milhorat, 1927; Boothby, Wilhelmj, and Wilson, 1929; Loewi, 1902; Reilly, Nolan, and Lusk, 1898). It is apparent from these data that more ingested glucose is recoverable in the urine in the depancreatized dog than in the phlorhizinized dog.

DISCUSSION

Low glucose yields from casein were obtained in the depancreatized dogs and in the duct-ligated phlorhizinized dogs. These were the animals in which there was a disturbance of the acinous pancreatic function. These low yields may be due to (*a*) impaired digestion, (*b*) utilization of a greater proportion of the sugar formed, or (*c*) incomplete conversion of the protein to glucose. In regard to the first possibility we believe that protein in 25 to 30 gm. quantities should be completely digested and absorbed (feces were rarely passed by these animals). Further, if one assumes that these animals convert casein to glucose to the same extent as intact phlorhizinized dogs, namely 49 per cent, and inserts such a yield in the Lusk equation which can be solved backwards for nitrogen intake, one obtains impossible answers. If the low yields were due to incomplete absorption of nitrogen, this procedure would reveal the true absorption. The fact that impossible answers are obtained proves that deficient digestion cannot be responsible for the low yields. These results further suggest that the depancreatized and duct-ligated animals do not convert casein to glucose to the extent of 49 per cent. That this is probably not the result of utilizing a greater proportion of the sugar formed is demonstrated by the fact that recovery of ingested glucose is greatest in the depancreatized dog. The only possibility left is that the low yields indicate impairment of gluconeogenesis, so that less sugar is formed from a given amount of protein. It is well known that the depancreatized dog receiving neither insulin nor raw pancreas very rapidly develops a fatty liver. Berg and Zucker (1931), Ralli, Rubin, and Present (1938), and Beazell and Schmidt[1] find similar changes follow complete ligation of the pancreatic ducts. Thus it is reasonable to consider that in the absence of acinous function of the pancreas, sufficient impairment of liver function occurs to depress gluconeogenesis.

Although impaired gluconeogenesis may explain the low yields on casein, it can hardly explain the low glucose to nitrogen ratio of the depancreatized dog after a 3 day fast, for the obvious reason that the duct-ligated dogs showed a high ratio in spite of their liver damage. However, it should be remembered that in contrast to the phlorhizinized dog, the long fasted depancreatized dog shows a progressively falling glucose to nitrogen ratio, and it is wholly possible that this phenomenon is a reflection of progressive liver damage, with consequent inability completely to convert protein to sugar. If it is true that the factor of liver damage is not operating in dogs fasted 3 days, why should it operate in the fed animals? Apparently the necessity of metabolizing a sudden influx of exogenous protein throws a strain on the liver which it cannot successfully withstand.

The low glucose to nitrogen ratio during fasting of the depancreatized dog may be due to oxidation of a greater proportion of sugar formed by gluconeogenesis, or to an inability to form sugar from unknown non-nitrogenous sources. We know of no satisfactory evidence which permits a choice between these alternatives.

SUMMARY

1. A comparative study has been made of the glucose to nitrogen ratios during fasting, the recovery of ingested glucose, and the conversion of protein to sugar in intact phlorhizinized dogs, in phlorhizinized dogs with ligated pancreatic ducts, and in depancreatized dogs.

2. There is a significant difference between the glucose to nitrogen ratios during fasting of depancreatized and phlorhizinized dogs.

---

[1] Beazell, J. A., and Schmidt, C. R., unpublished data.

3. A greater proportion of ingested sugar is recoverable in the urine of depancreatized dogs than in that of phlorhizinized dogs.

4. Depancreatized dogs and duct-ligated phlorhizinized dogs give low glucose yields from ingested protein. Since impaired digestion cannot account for this finding, it is suggested that impaired gluconeogenesis in the liver may be responsible.

BIBLIOGRAPHY

Allard, E., *Arch. exp. Path. u. Pharmakol.*, **59,** 111 (1908).

Barker, S. B., Chambers, W. H., and Dann, M., *J. Biol. Chem.*, **118,** 177 (1937).

Benedict, S. R., *J. Am. Med. Assn.*, **57,** 1193 (1911).

Berg, B. N., and Zucker, T. F., *Proc. Soc. Exp. Biol. and Med.*, **29,** 68 (1931).

Boothby, W. M., Wilhelmj, C. M., and Wilson, H. E. C., *J. Biol. Chem.*, **83,** 657 (1929).

Coolen, F., *Arch. pharmacod.*, **1,** 267 (1895).

Cori, C. F., and Cori, G. T., in Harrow, B., and Sherwin, C. P., A textbook of biochemistry, Philadelphia and London (1935).

Csonka, F. A., *J. Biol. Chem.*, **20,** 539 (1915).

Dann, M., *Yale J. Biol. and Med.*, **5,** 359 (1933).

Dann, M., Chambers, W. H., and Lusk, G., *J. Biol. Chem.*, **94,** 511 (1931–32).

Deuel, H. J., Jr., and Chambers, W. H., *J. Biol. Chem.*, **65,** 7 (1925).

Deuel, H. J., Jr., Wilson, H. E. C., and Milhorat, A. T., *J. Biol. Chem.*, **74,** 265 (1927).

Janney, N. W., *J. Biol. Chem.*, **20,** 321 (1915).

Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46,** 2066 (1924).

Loewi, O., *Arch. exp. Path. u. Pharmakol.*, **47,** 68 (1902).

Minkowski, O., *Arch. exp. Path. u. Pharmakol.*, **31,** 85 (1893).

Moorhouse, V. H. K., Patterson, S. W., and Stephenson, M., *Biochem. J.*, **9,** 171 (1915).

Nash, T. P., Jr., *J. Biol. Chem.*, **66,** 869 (1925).

Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, **55,** 757 (1923).

Olmsted, W. H., *J. Biol. Chem.*, **41,** 45 (1920).

Ralli, E. P., Canzanelli, A., and Rapport, D., *Am. J. Physiol.*, **96,** 331 (1931).

Ralli, E. P., Rubin, S. H., and Present, C. H., *Am. J. Physiol.*, **122,** 43 (1938).

Reilly, F. H., Nolan, F. W., and Lusk, G., *Am. J. Physiol.*, **1,** 395 (1898).

Ringer, A. I., *J. Biol. Chem.*, **12,** 431 (1912).

Ringer, A. I., and Frankel, E. M., *J. Biol. Chem.*, **18,** 81 (1914).

Ringer, M., *J. Biol. Chem.*, **58,** 483 (1923–24).

Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, **24,** 23 (1916).

Wierzuchowski, M., *J. Biol. Chem.*, **73,** 417 (1927).

THE COMPOSITION OF KERATINS

THE AMINO ACID COMPOSITION OF HAIR, WOOL, HORN, AND OTHER EUKERATINS

By RICHARD J. BLOCK

With the Cooperation of Diana Bolling, Florence C. Brand, and Arnold Schein

(From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York)

(Received for publication, January 23, 1939)

Previous investigations on the amino acid composition of keratins have indicated that this class of proteins may be subdivided into two groups: eukeratins and pseudokeratins (1). Both are characterized by their insolubility in aqueous and organic solvents and by a relatively high degree of resistance towards enzymatic hydrolysis, but the eukeratins appear to be differentiated by a constant proportion of histidine, lysine, and arginine which are present in the molecular ratios of approximately 1:4:12. The constancy of this ratio in proteins of widely different biological origin led to the view (*cf.* (2)) that the basic amino acids may be of some special importance in the development of tissue proteins. It is possible that these three amino acids have a directive influence on protein synthesis in the organism. Thus the recognized similarities in the gross structure of the eukeratins might depend upon the proportions of histidine, lysine, and arginine, while the variations in the individual keratins are the result of changes in the amounts of the non-basic amino acids.

In contrast to the relative constancy of arginine, histidine, and lysine, the variability in the amount of cystine present in eukeratins was early demonstrated by Buchtala (3) who isolated 14 to 15 per cent of cystine from human hair, 8 per cent from horse hair, 3 per cent from horse's hoofs, etc. Comparative biochemical investigations on the amino acid composition of eukeratins are lacking and it was deemed of interest to extend our knowledge of these proteins.

EXPERIMENTAL

The preparation of the tissues for analysis was carried out by the methods described previously (4) except that the animal hairs were not subjected to digestion by pepsin and trypsin.

All chemical analyses were repeated two or more times. Total nitrogen was determined by the macro-Kjeldahl method with Cu and Se as catalysts and sulfur by the Parr bomb method. The basic amino acids were isolated from 2.5 gm. of protein by silver precipitation (5) and the purity of the resulting flavianates, nitranilates, and picrates was checked by analyses and melting points. Cystine was determined by Miller and du Vigneaud's procedure (6); tyrosine and tryptophane were estimated by a modification of Folin's method (*cf.* (5)). Phenylalanine was determined by the dinitrobenzene procedure (5) and glycine was isolated by a modification of the method of Bergmann and Fox (*cf.* (5)).

It should be recognized that the methods used for the estimation of the eight amino acids are not of equal precision. The advantages and drawbacks of the above procedures have been discussed recently (5). However, it is the writer's opinion that all are of value in a comparative investigation although they may not be entirely reliable as a means of estimating absolute amounts of amino acids.

Results

The analytical data are summarized in Table I which also includes determinations made by Calvery (7), Hess (8), and Block and Lewis (9). The earlier results of the author have been confirmed in practically all instances. In each case, however, the more recent analysis is reported.

Arginine, Histidine, and Lysine—Of the eight amino acids investigated, arginine, which can be determined with a relatively high degree of accuracy, is the most constant and was, therefore, chosen as the base-line for the calculation of the molecular ratios. The outstanding fact apparent from Table I is the relative constancy of histidine, lysine, and arginine to each other. These three amino acids are in the ratio 1:4:12 or 1:5:12 in all cases, except for goat hair which yields somewhat more lysine.

TABLE I

Amino Acid Composition of Some Eukeratins

Protein	Nitrogen	Sulfur	Histidine	Lysine	Arginine	Cystine	Tyrosine	Tryptophane	Phenylalanine	Glycine	Molecular ratios							
											Histidine to	Lysine to	Arginine to	Cysteine* to	Tyrosine to	Tryptophane to	Phenylalanine to	Glycine
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*								
Human hair	15.4	5.0	0.6	2.5	8.0	15.5	3.0	0.7	2.6	4.3	1	4	12	34	4	1	4	15
Chimpanzee hair (5)†	16.7	4.3	0.6	2.0	8.1	15.5	3.3	1.4			1	4	12	33	5	2		
Goat hair	16.2	3.1	0.7	3.2	8.1	8.9	3.0	0.9	4.6	6.3	1	6	12	19	4	1	7	22
Cow " (5)	15.3	3.7	0.7	2.0	7.5	13.4	3.3	1.4	3.9	10.3	1	4	12	27	5	2	7	38
Lamb wool	15.4	3.6	0.7	2.5	8.7	13.1	4.5	0.7	4.0	6.5	1	4	12	26	6	1	6	21
Camel "	15.1	3.1	0.6	2.7	8.6	11.0	3.1	0.8	4.1	9.2	1	4	12	22	4	1	6	30
Cattle horn	16.1	2.6	0.6	2.4	8.6	8.2	3.7	0.7	4.0	9.8	1	4	12	17	5	1	6	32
Rhinoceros horn	15.6	2.3	0.6	2.6	8.2	8.7	8.6	1.7	5.0	7.4	1	5	12	18	12	2	8	25
Finger nails (7)	14.9	3.8	0.5	2.6	8.5	12.0	3.0	1.1	2.5		1	4	12	25	4	1	4	
Porcupine quills	15.8	3.0	0.6	2.6	7.6	9.4	3.3	0.9	3.6	5.7	1	5	12	18	5	1	6	21
Echidna quills	15.2	3.8	0.5	1.8	6.8	11.9	9.1	2.2	6.8		1	4	12	30	15	3	13	
Hen feathers	15.5	2.3	0.3	1.6	6.0	6.8	2.2	0.7	5.3	9.5	1	4	12	17	4	1	11	44
Snake skin	15.2	2.2	0.4	1.9	5.4	6.6	5.2	0.9	3.9	13.1	1	5	12	21	11	2	9	67
Egg shell membrane (4)	16.6	3.8	0.9	3.7	9.9	12.7	2.5	2.6	2.0		1	5	12	22	3	3	3	

* The cystine values have been calculated as cysteine.

† Bibliographic reference number.

This increased amount of lysine does not appear to be the result of an analytical error but probably represents an actual difference in the composition of this tissue protein. The relatively small amount of histidine isolated from feathers may be due to analytical difficulties.

Sulfur and Cystine—In confirmation of the older literature, the cystine (3) and sulfur (10) contents of the eukeratins are variable. Primate hair (human and chimpanzee) contains more cystine than any other keratin (*cf.* (9)). The ratio of cystine to arginine in cow hair and echidna spines is almost as great as in primate hair, although the actual amounts of cystine in these proteins are less. Goat hair, cattle horn, rhinoceros horn, porcupine quills, and hen feathers yield comparatively small amounts of cystine. It is known that health and disease (8), as well as diet, can influence the cystine and sulfur content of hair (9, 11, 12), but the old claims of von Bibra (10) and of Fourcroy and Vauquelin (13) that red, blond, and white hair contains more sulfur than black hair are probably erroneous (*cf.* (9)).

Tyrosine and Tryptophane—The tyrosine contents of the eukeratins reported in this investigation are about 3 per cent, except in the cases of rhinoceros horn and echidna spines which yield approximately 9 per cent of this amino acid and snake skin which contains over 5 per cent. Rhinoceros horn and echidna spines are remarkably hard substances. Egg shell membrane is the only protein reported in which the content of tryptophane equals that of tyrosine. This observation of Calvery (7) has been confirmed by the author.

Phenylalanine—Variations in the amount of phenylalanine present in several eukeratins were indicated by the earlier investigations of Fischer and Dörpinghaus (14), Abderhalden and Voitinovici (15), and Lissizin (16). The analytical results reported in Table I, all of which are those of the writer, confirm these impressions, for the values range from 2.0 per cent of phenylalanine in egg shell membrane to 6.8 per cent in echidna spines. The values of 2.5 per cent in human finger nails and of 2.6 per cent in human hair are of interest.

Glycine—The eukeratins are relatively rich in glycine. The amounts present in hen feathers and snake skin are especially high. The isolation of over 13 per cent of glycine from the latter protein indicates some similarity to the *pseudokeratins*, gorgonin and spongin, which contain approximately 14 per cent of glycine (4). Buchtala (3) claimed that the amino acid composition of human hair and sheep wool differs principally in that the former has a considerably greater proportion of glycine. Our results do not support this opinion, for when the same method of determination was employed, human hair yielded 4.3 per cent, while wool contained approximately 6.5 per cent of glycine.

SUMMARY

1. The amounts of nitrogen, sulfur, histidine, lysine, arginine, cystine, tyrosine, tryptophane, phenylalanine, and glycine were determined in human hair, goat hair, lamb wool, camel wool, cattle horn, rhinoceros horn, finger nails, porcupine quills, echidna spines, hen feathers, and snake skin.

2. In confirmation of earlier findings, the molecular ratios of histidine to lysine to arginine in these proteins remained relatively constant. In contrast, decided variations in the actual and relative amounts of cystine, tyrosine, tryptophane, phenylalanine, and glycine were noted.

3. Similarities and differences in the amino acid composition of fourteen eukeratins are given in Table I. Some salient points are (*a*) the relatively high content of sulfur and cystine in human and chimpanzee hair, of phenylalanine in echidna quills, of glycine in feathers and snake skin, of tyrosine in echidna spines and rhinoceros horn, and of tryptophane in egg shell membrane and echidna spines; (*b*) the relatively small amounts of cystine in hen feathers and cattle horn, of tyrosine in egg shell membrane, of phenylalanine in egg shell membrane and finger nails, and of glycine in human hair.

4. The data given in this paper are interpreted as lending support to previous suggestions concerning the special function of histidine, arginine, and lysine, particularly the first two, both in the genetic development of tissue proteins and in imparting to a protein its group or general characteristics. Variations in the other amino acids are probably responsible for specific individual differences. The stress laid on the constancy of the basic amino acids should not be understood as implying that these three acids alone have a unique importance in protein structure, although

they may have in protein synthesis. The variability of the other amino acids and especially the changes in the cystine content of nails and hair in health and disease are of great significance. Further investigations on the comparative composition of the eukeratins are contemplated, for it is this way alone which will show whether or not amino acids, other than the basic amino acids, are present in the eukeratins in definite proportions.

Addendum—The discrepancies between the values reported for total sulfur and cystine in gorgonin and spongin (4) are due to the failure to correct for the large amount of ash. Similar analytical results were obtained in 1931 as shown by the data given in Column 2 of Table I (4). When the correction for ash is made, the pseudokeratins, like the eukeratins, appear to contain the greatest proportion, if not all, of their organic sulfur in the form of cystine sulfur.

BIBLIOGRAPHY

1. Block, R. J., *J. Biol. Chem.*, **121,** 761 (1937).
2. Schmidt, C. L. A., The chemistry of the proteins and amino acids, Springfield (1938).
3. Buchtala, H., *Z. physiol. Chem.*, **85,** 241, 246, 335 (1913).
4. Block, R. J., and Bolling, D., *J. Biol. Chem.*, **127,** 685 (1939).
5. Block, R. J., The determination of the amino acids, Minneapolis, revised edition (1938).
6. Miller, G. L., and du Vigneaud, V., *J. Biol. Chem.*, **118,** 101 (1937).
7. Calvery, H. O., *J. Biol. Chem.*, **100,** 183 (1933).
8. Hess, W. C., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **109,** p. xliii (1935).
9. Block, W. D., and Lewis, H. B., *J. Biol. Chem.*, **125,** 561 (1938).
10. von Bibra, F., *Ann. Chem. u. Pharm.*, **96,** 289 (1855).
11. Lightbody, H. D, and Lewis, H. B., *J. Biol. Chem.*, **82,** 485, 663 (1929).
12. Heard, E. V., and Lewis, H. B., *J. Biol. Chem.*, **123,** 203 (1938).
13. Fourcroy, M., and Vauquelin, L.-N., *Ann. chim.*, **58,** 41 (1806).
14. Fischer, E., and Dörpinghaus, T., *Z. physiol. Chem.*, **36,** 462 (1902).
15. Abderhalden, E., and Voitinovici, A., *Z. physiol. Chem.*, **52,** 348 (1907).
16. Lissizin, T., *Z. physiol. Chem.*, **62,** 226 (1909).

THE OCCURRENCE OF CYCLIZED VITAMIN A IN FISH LIVER OILS*

BY NORRIS D. EMBREE

(*From the Laboratories of Distillation Products, Inc., Rochester, New York*)

(Received for publication, December 15, 1938)

From a vitamin A concentrate prepared from halibut liver oil, Castle, Gillam, Heilbron, and Thompson (1) separated a yellow substance with an ultraviolet absorption spectrum similar to that shown in Fig. 1. The maxima of the three absorption bands occurred at 348, 369, and 389 mμ. The concentrate gave a blue color with antimony trichloride very similar to that of vitamin A, with a maximum absorption at 624 mμ and much less absorption at 579 mμ. Heilbron, Heslop, Morton, Rea, and Drummond (2) found that the spectra of the most volatile fractions from a prolonged vacuum distillation of a very potent vitamin A concentrate had a fine structure corresponding to these bands. Pritchard, Wilkinson, Edisbury, and Morton (3) found that the fraction of a sample of mammalian liver concentrate, which was insoluble in 83 per cent alcohol, had a spectrum with slight maxima also at 350, 367, and 390 mμ.

In these laboratories a substance with a similar absorption spectrum has been noted in the low boiling fractions of fish liver oils distilled in the molecular still. Fig. 1 shows the absorption spectrum of a fraction from the redistillation of a tuna liver oil distillate. The absorption maxima occur at 350, 368, and 379 mμ. $E_{1\,\text{cm.}}^{1\%}$ (368 mμ) = 1385.

Because the substance of most interest at the time of these distillations was vitamin A, the interference of this triple banded material with assays made by the Hilger vitameter earned it the temporary name of "spurious A."

A material with a spectrum similar to that of spurious A has been prepared by Edisbury, Gillam, Heilbron, and Morton (4). Vitamin A was "cyclized" by them by treating it with N/30 hydrogen chloride in absolute alcohol. The "cyclized vitamin A" had absorption maxima at 333, 350, 369, and 392 mμ. "Cyclization" of vitamin A alcohol concentrates[1] by their method has been carried out several times in these laboratories, and each time the cyclized material had only the three absorption maxima characteristic of spurious A (Fig. 2). The crude cyclized material was

---

* Communication No. 7 from the Laboratories of Distillation Products, Inc.

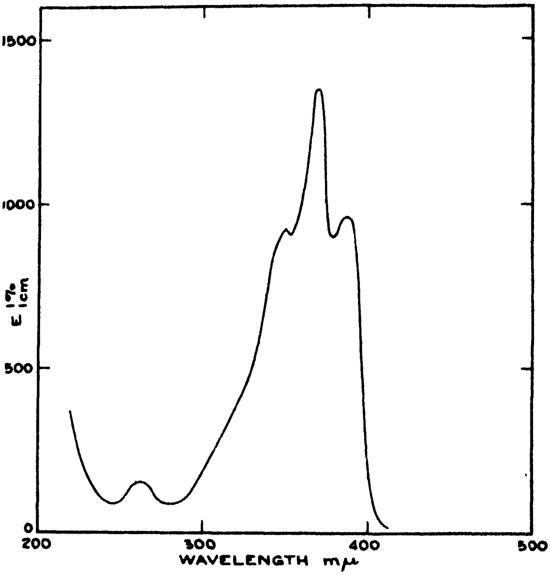


FIG. 1. The ultraviolet absorption spectrum of a concentrate of spurious A from tuna liver oil.

---

[1] Vitamin A ester concentrates, such as high potency distillates of halibut liver oil, may be cyclized by the same method. But these materials must be exposed to the alcoholic hydrogen chloride for 4 hours instead of 20 minutes at room temperature. The vitamin A activity of the material which Pritchard *et al.* (3) found in the petroleum ether solution of a vitamin A alcohol concentrate after the vitamin A alcohol had been extracted with 83 per cent alcohol might well have been due to small amounts of unsaponified vitamin A ester. The ester will not dissolve in 83 per cent alcohol, and like Pritchard's material, will not produce cyclized vitamin A with 20 minutes exposure to alcoholic hydrogen chloride.

purified by distillation, and the first fractions had the same spectrum as that of the spurious A concentrate shown in Fig. 1. The later fractions had spectra showing the presence of more and more unchanged vitamin A. The spectrum of the most potent fraction so far obtained with E (368 mμ) = 1442 is also shown in Fig. 2. Evidently the band at 333 mμ described by Edisbury *et al.* was due to vitamin A still present in the mixture. In the cyclizations studied so far, the extinction coefficient at 368 mμ of the cyclized vitamin A formed is 100 to 110 per cent of the extinction coefficient of the vitamin A converted. This suggests the possibility that the maximum extinction coefficient of cyclized vitamin A is about the same as that of vitamin A, and, therefore, the amounts present of the two materials may be compared by comparing their maximum extinction coefficients. A method for estimating the individual extinction coefficients in a mixture of both will be discussed later.

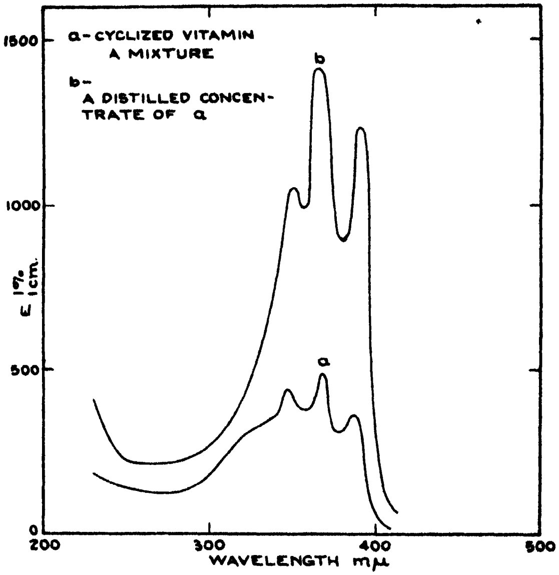


FIG. 2. The ultraviolet absorption spectra of preparations of cyclized vitamin A. (*a*) represents a crude product resulting from treatment of vitamin A with N/30 alcoholic hydrogen chloride; (*b*), a distillate of the crude product.

Some of the physical and chemical properties of spurious A and cyclized vitamin A were studied in the attempt to determine their nature and their possible identity.

Both substances in the concentrated fractions were mobile, orange-red liquids with, as has already been mentioned, the same maxima of ultraviolet absorption and about the same extinction coefficient in the highest concentrates of each obtained so far.

Neither substance can be extracted from a petroleum ether solution with 83 per cent alcohol.

Both substances are very unstable. Great care is needed to prevent the oxidation of concentrated samples.

TABLE I

Analytical Distillation of Spurious A Concentrate

Fraction No.	Temperature	Pressure of residual gas	Per cent distilled	E (328 mμ) by vitameter
	°C.	*microns*		
1	80	3.8	3.2	38.3
2	90	3.8	2.8	61.5
3	100	3.8	3.4	78.5
4	110	3.8	2.8	64.8
5	120	3.3	3.9	34.3
6	130	3.3	4.8	13.1
7	140	2.8	6.2	4.5
8	150	3.0	6.1	1.8

The elimination curve, the graph against the temperature of distillation of the percentage yields at each temperature from a mixture of synthetic glycerides by analytical molecular distillation (5, 6), was determined for each material and was found to be the same. The maxima of the curves occurred at about 100°, or some 20° lower than for vitamin A. This showed that the ratios of the vapor pressure to the square root of the molecular weight were the same for the two substances. The data for the elimination curve for spurious A are given in the next two paragraphs and in Table I.

Analytical Distillation No. ES-25—The distilland was composed of 2 cc. of a spurious A concentrate, 27 cc. of the high boiling fraction (free from vitamin A) from fish liver oil, and 30 cc. of constant yield oil.

The mixture was treated in a small cyclic still having a distilling column 2.4 cm. in diameter and 7.0 cm. in length. The rate of circulation was set so that the reservoir was emptied in 9 minutes and another minute was allowed for the oil in the connecting tubing to be replaced, thus giving a 10 minute cycle.

The complete ultraviolet spectra of Fractions 4 and 6 (Table I) were examined and found to be typical of spurious or cyclized vitamin A. Accordingly, the extinction coefficients at 328 mμ, as determined by the vitameter, were considered to be a measure of the amount of cyclized vitamin A present. The peak of the elimination curve for vitamin A alcohol, when determined in a similar manner on the same still, is about 120°.

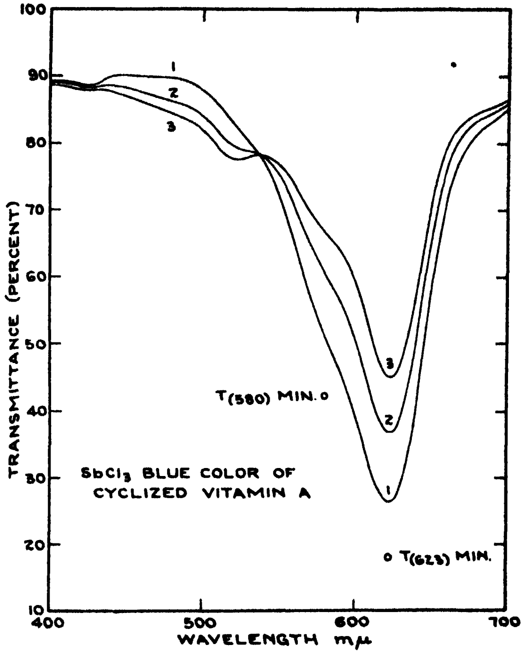


FIG. 3. The transmission spectrum of the antimony trichloride blue color of a cyclized vitamin A concentrate. O = minimum transmittance of a fresh solution. Curves 1, 2, and 3 represent transmission curves drawn in quick succession.

Edisbury *et al.* (4) state that with antimony trichloride in chloroform, cyclized vitamin A reacts to give the same color that vitamin A gives. We find that purified samples of cyclized vitamin A prepared by distillation have this same property. The concentrates of naturally occurring spurious A act similarly. The reaction gives a greenish blue solution with a maximum absorption at 623 mμ.

The transmission spectra of the blue colors were measured in a Hardy (7) recording spectrophotometer made by the General Electric Company. The transmission curves for a distilled cyclized vitamin A having E (328 mμ) = 592 and E (368 mμ) = 1318 are shown in Fig. 3. To prepare the test mixture for the recording photometer, 10 cc. of a saturated (at 20°) solution of SbCl$_3$ in chloroform were added to the absorption cell in which had already been added 1 cc. of 0.00456 per cent solution of the cyclized vitamin A in chloroform. The wave-length scale had been set at 580 mμ. In 5 seconds the transmission read reached a minimum of 42.3 per cent and then started to increase. The reaction was repeated with the wave-length scale at 623 mμ. The transmission reached a minimum of 18.0 per cent and then began to rise. The wave-length scale was then set back to 400 mμ and the instrument allowed to trace out the transmission curve three times. The quickness of the fading is shown by the extinction coefficients calculated at the times when the wave-lengths 580 and 623 mμ were reached (Table II).

TABLE II

Extinction Coefficients of SbCl$_3$ Blue Color of Cyclized Vitamin A Concentrate

Time	E (580 mμ)	E (623 mμ)
Maximum	1020	1800
1 min. 15 sec.	706	
1 " 30 "		1430
5 " 25 "	527	
5 " 40 "		1050
9 " 40 "	425	
10 " 5 "		860

Each of the curves had a sharp minimum at 623 mμ. The 580 mμ band was almost hidden by the 623 mμ band. The behavior is very much like the reaction with vitamin A concentrates, except that the latter have transmission minima at 620 to 622 mμ and usually show no sign of a band near 580 mμ. The ratio of *E* (620 mμ) to *E* (328 mμ) ranges from 3 to 4 for most highly concentrated spurious A or cyclized vitamin A preparations, while it is about 3 for many vitamin A concentrates. Thus, the error in assaying vitamin A samples which contain spurious A or cyclized vitamin A by the $SbCl_3$ color method may be even greater than when the ultraviolet absorption at 328 mμ is used.

The two substances do not form crystalline addition products with picric acid. When varying amounts of the concentrates were added to a saturated solution of picric acid in alcohol, the solutions turned dark, but no crystals were formed. Hydrocarbons with similar absorption spectra described by Hausser, Kuhn, and Smakula (8) did form these crystalline addition products very easily.

The similar physical and chemical properties of spurious A and cyclized vitamin A indicate that it is very unlikely that they are not identical. It will be assumed now that they are the same substance. The name cyclized vitamin A is rather a poor choice, because the nature of the cyclization has not yet been established. However, 1,6-dimethylnaphthalene has been found by Heilbron, Morton, and Webster (9) in material formed by selenium dehydrogenation of a vitamin A concentrate and of a cyclized vitamin A concentrate prepared by the method of Edisbury *et al.* (4), which probably contained appreciable amounts of unchanged vitamin A. The dimethylnaphthalene ring may have been formed from the cyclized vitamin A during the dehydrogenation or may even have come from vitamin A and not cyclized vitamin A. Since either name is only useful until the substance is actually identified, the name cyclized vitamin A will be used because it has already been referred to by that name in the literature.

As a rule, cyclized vitamin A is found along with vitamin A so that the absorption spectrum of the material studied is a combination of the two. Therefore, a method for estimating the relative amounts of the two substances is quite valuable. The "purest" spectrum, that is the one with the highest ratio of *E* (368 mμ) to *E* (326 mμ), of cyclized vitamin A that we have prepared is that shown in Fig. 1. This ratio is 3.04. The average ratio of E (368 mμ) to E (326 mμ) for many samples of vitamin A concentrates made by molecular distillation from fish liver oils in these laboratories is 1:3.84. In our experience the maximum absorption is at 326 mμ for vitamin A alcohol concentrates, and it is with this form of the vitamin that the cyclized vitamin A usually occurs. If E_{326}^{V} is allowed to represent the extinction coefficient at 326 mμ due to the vitamin A present, E_{368}^{V} for the extinction coefficient at 368 mμ for the vitamin, and correspondingly E_{326}^{C} and E_{368}^{C} for the cyclized vitamin, and E_{326}^{T} and E_{368}^{T} for the total observed extinction coefficients, then by a solution of the simultaneous equations involved, the fraction of the absorption at 326 mμ due to the vitamin is

$$\frac{E_{326}^{V}}{E_{326}^{T}} = 1.094 - 0.359\frac{E_{368}^{T}}{E_{326}^{T}} \tag{1}$$

The fraction of the absorption at 326 mμ due to the cyclized vitamin is

$$\frac{E_{326}^{C}}{E_{326}^{T}} = 1 - \frac{E_{326}^{V}}{E_{326}^{T}} \tag{2}$$

These equations are not offered with the purpose of making exact calculations, but are expected to be useful in estimating the amounts of the materials involved.

A test of the equation was made on one of the first samples made from tuna liver oil, which had the characteristic spectrum of cyclized vitamin A. The value for E (326 mμ) of the sample was 125 and that for E (368 mμ) was 309, which, by Equation 1, would predict that the portion of E (326 mμ) due to the vitamin was 27. The sample was biologically assayed[2] against U.S.P. reference oil and found to contain 75,000 units of vitamin A per gm. In our experience, vitamin A concentrates which have been processed to remove other materials which absorb in the ultraviolet have a ratio of E (326 to 328 mμ) to U.S.P. units per gm. of about 2110. Thus, the vitamin A contained in the sample could be represented by a vitamin A absorption curve with E (326 mμ) of about 30, which compares quite favorably with the predicted value of 27. This rough check helps to validate Equation 1 and, at the same time, indicates that the cyclized vitamin A has no vitamin A potency for rats. Further studies of the biological properties of cyclized vitamin A are in progress.

---

[2] Assayed by H. J. Cannon of the Laboratory of Vitamin Technology, Chicago.

Because cyclized vitamin A had not been reported before as a common constituent of fish liver oils, the possibility was considered that it might be formed by the thermal decomposition of vitamin A during distillation. Such a decomposition was reported when the vitamin is heated or distilled by Heilbron *et al.* (2). Vitamin A ester and alcohol concentrates prepared from tuna liver oil were heated at 200° and the spectra examined. In neither case was cyclized vitamin A formed. At this temperature the vitamin A ester concentrate had a half life of about 18 minutes (see Figs.

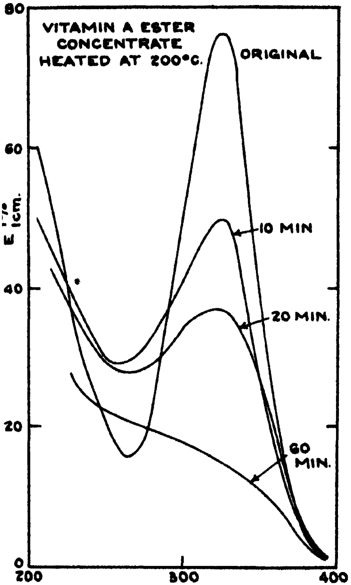


Fig. 4

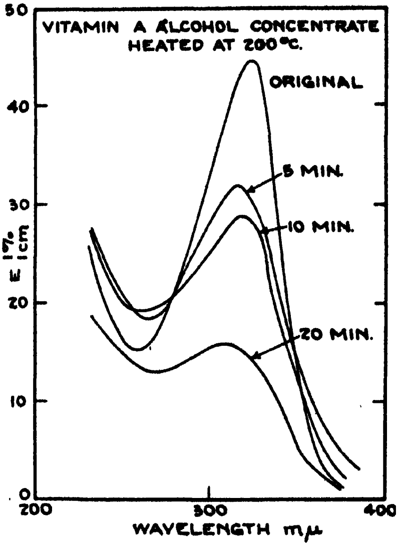


Fig. 5

Fig. 4. The effect of heating a vitamin A ester concentrate at 200°. Absorption spectra are shown for the original material and for the products after being heated for 10, 20, and 60 minutes.

Fig. 5. The effect of heating a vitamin A alcohol concentrate at 200°. Absorption spectra of the original material and the products after being heated for 5, 10, and 20 minutes.

4 and 5) and the alcohol concentrate had a half life of about 7 minutes.

Possibly, too, the cyclized vitamin A might have been produced by action at the distillation temperatures of the free fatty acids in the oils. When a vitamin A ester and an alcohol concentrate were heated for 3 minutes at 200° in the presence of 80 per cent oleic acid, some cyclized vitamin A was formed (Figs. 6 and 7). However, when 10 per cent oleic acid (Fig. 8) was used, little cyclized vitamin A was formed, although the half life of the vitamin A ester concentrate was cut down to about 7 minutes.

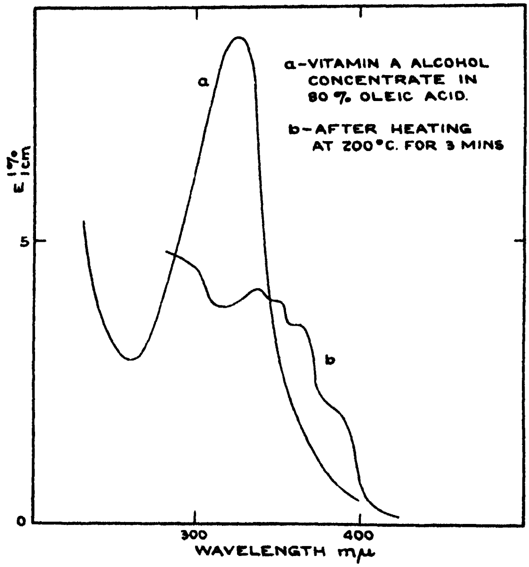


FIG. 6. The effect of heating a vitamin A alcohol concentrate with 80 per cent oleic acid. (*a*) represents the absorption spectrum before heating; (*b*), absorption spectrum after being heated at 200° for 3 minutes.

Fractions from fish oils, containing cyclized vitamin A, that we have studied were distilled with only a few seconds' exposure to temperatures well under 200° from oils with free fatty acid contents varying from 0.01 to 0.5 per cent as oleic acid. The vitamin A yields were around 100 per cent, so there can be little doubt that substantially all the cyclized vitamin A found in the distillates was present in the original oil.

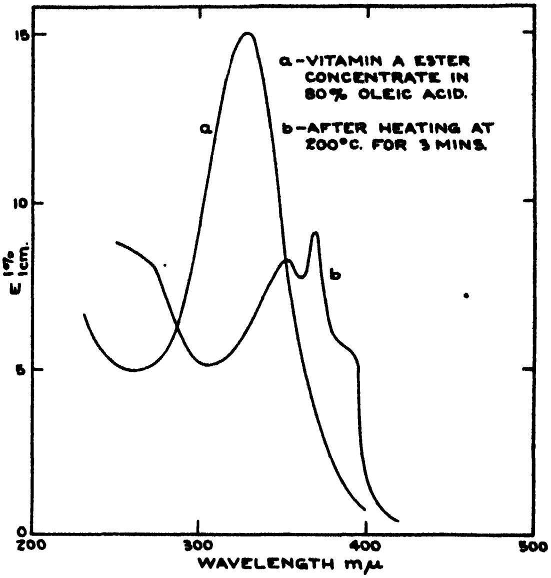


FIG. 7. The effect of heating a vitamin A ester concentrate with 80 per cent oleic acid. (*a*) represents the absorption spectrum before heating; (*b*), absorption spectrum after being heated at 200° for 3 minutes.

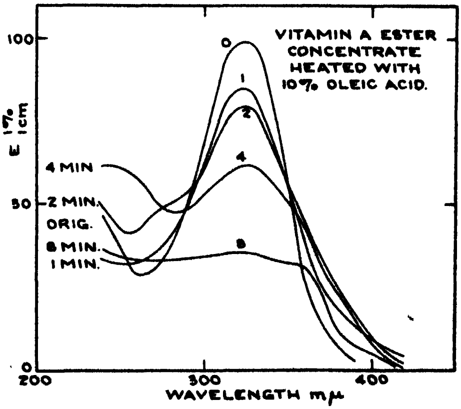


FIG. 8. The effect of heating a vitamin A ester concentrate with 10 per cent oleic acid. Absorption spectra are shown for the original material and for the products after being heated at 200° for 1, 2, 4, and 8 minutes.

Molecular distillations of samples of halibut, pollack, and cod liver oils have been carried out. The lowest boiling fractions, like those from tuna liver oil, show the presence of cyclized vitamin A. Its concentration is often about 2 per cent of that of vitamin A.

The author wishes to express his thanks to Kenneth C. D. Hickman for many valuable suggestions, to other chemists of Distillation Products, Inc., for their technical assistance, and to the physics department of the Research Laboratories of the Eastman Kodak Company for the measurement of absorption spectra.

SUMMARY

Cyclized vitamin A, the material formed by treatment of vitamin A with HCl in alcohol, has been found to occur naturally in tuna and other fish liver oils. It appears to have no growth-promoting power, but has considerable ultraviolet absorption at 328 mμ and gives practically the same $SbCl_3$ reaction as vitamin A.

BIBLIOGRAPHY

1. Castle, D. C., Gillam, A. E., Heilbron, I. M., and Thompson, H. W., *Biochem. J.*, **28**, 1702 (1934).
2. Heilbron, I. M., Heslop, R. N., Morton, R. A., Rea, J. L., and Drummond, J. C., *Biochem. J.*, **26**, 1178 (1932).
3. Pritchard, H., Wilkinson, H., Edisbury, J. R., and Morton, R. A., *Biochem. J.*, **31**, 258 (1937).
4. Edisbury, J. R., Gillam, A. E., Heilbron, I. M., and Morton, R. A., *Biochem. J.*, **26**, 1164 (1932).
5. Hickman, K. C. D., *Ind. and Eng. Chem.*, **29**, 968 (1937).
6. Embree, N. D., *Ind. and Eng. Chem.*, **29**, 975 (1937).
7. Hardy, A. C., *J. Optical Soc. Am.*, **25**, 305 (1935).
8. Hausser, K. W., Kuhn, R., and Smakula, A., *Z. physik. Chem., Abt. B*, **29**, 388 (1935).
9. Heilbron, I. M., Morton, R. A., and Webster, E. T., *Biochem. J.*, **26**, 1194 (1932).

STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

XI. THE DETOXICATION OF BENZYL CHLORIDE, BENZYL ALCOHOL, BENZALDEHYDE, AND S-BENZYLHOMOCYSTEINE IN THE RABBIT AND RAT*

By JAKOB A. STEKOL

(*From the Department of Chemistry, Fordham University, New York*)

(Received for publication, January 4, 1939)

In an earlier paper we reported that benzyl chloride, when fed to dogs, yielded N-acetyl-S-benzylcysteine in the urine. The same mercapturic acid was formed from S-benzylcysteine in the organism of the rat, rabbit, and dog (1).

The present report deals with detoxication of benzyl alcohol, which may conceivably be an intermediate which is formed from benzyl chloride in the animal body before the synthesis of benzylmercapturic acid occurs. Should this assumption be true, one could then expect benzylmercapturic acid to appear in the urine of animals to which benzyl alcohol was fed or injected. On the other hand, if benzyl alcohol could be demonstrated to be metabolized and yield in the urine products other than the expected benzylmercapturic acid, then this aromatic alcohol could hardly be considered as a probable intermediate in the synthesis of benzylmercapturic acid from benzyl chloride. We therefore administered benzyl alcohol to rabbits and rats and isolated from the urine of these animals the detoxication products. In addition, benzaldehyde was similarly studied, as it appeared possible that this substance might arise from benzyl alcohol on oxidation of the latter.

Another point which we wished to investigate in the present work was the possible rôle of homocysteine in the detoxication of benzyl chloride. In experiments with rats fed diets deficient in the sulfur-containing amino acids, homocystine can supplement a cystine-deficient or methionine-deficient diet as effectively as cystine or methionine, respectively, for purposes of growth (2, 3). These findings may be interpreted as indicating the conversion of homocystine into cystine or methionine respectively. It appeared possible that benzyl chloride, when fed to animals, combined first with homocysteine and the resulting S-benzylhomocysteine then underwent conversion to S-benzylcysteine and subsequently to benzylmercapturic acid. Should this be true, the administration of S-benzylhomocysteine to animals would yield in the urine N-acetyl-S-benzylcysteine.

---

* The results on S-benzylhomocysteine were reported in a preliminary paper at the meeting of the American Chemical Society, at Milwaukee, in September, 1938.

It appeared desirable also to establish the synthesis of benzylmercapturic acid from benzyl chloride in the rabbit and rat.

The experiments presented here are concerned with the fate of benzyl alcohol, benzaldehyde, benzyl chloride, and S-benzylhomocysteine in the rabbit and rat.

EXPERIMENTAL

Four albino rabbits of 2 to 3 kilos in weight and six albino rats of 200 to 300 gm. in weight were used. The animals were kept in individual metabolism cages and fed Purina Chow and water *ad libitum* unless indicated otherwise. The urine was preserved with toluene.

dl-S-Benzylhomocysteine was prepared from *dl*-homocystine (4); the latter was synthesized from *dl*-methionine (5). Benzaldehyde and benzyl alcohol were commercial products. All the substances were analytically pure. The substances isolated from the urine were analyzed for carbon and hydrogen (Pregl), nitrogen (micro-Dumas), and acetyl (Elek and Harte (6)). The melting points of the substances were not corrected.

Experiments with Benzyl Alcohol and Benzaldehyde—1.0 gm. each of benzyl alcohol and of benzaldehyde was injected subcutaneously in three portions 2 hours apart into rabbits, two animals being injected with each of the substances studied. 24 hour samples of urine from each rabbit were collected separately, and the following procedure for the isolation of the detoxication products was applied to each urine.

The urine was neutralized with HCl and evaporated *in vacuo* at 38–40° to a syrup, acidified with HCl to Congo red paper, and extracted with several portions of ethyl acetate. The extract was then evaporated *in vacuo* at 38–40° to dryness, and the residue was dissolved in boiling water. On cooling, a yellowish precipitate separated. It was centrifuged off, again dissolved in boiling water, and decolorized with norit A. On cooling the filtrate, shiny short needles crystallized. The product was recrystallized from water several times until perfectly colorless crystals were obtained. The yield was 300 to 400 mg. per 1.0 gm. of benzyl alcohol or benzaldehyde administered. The products were dried *in vacuo* over H_2SO_4, and on analysis proved to be identical with hippuric acid.

Compound injected	C	H	N	M.p. °C.
Benzyl alcohol. Found	60.0	5.20	7.80	187–188
Benzaldehyde. "	60.28	5.15	7.82	186–187
Calculated for hippuric acid	60.30	5.03	7.82	187–188

500 mg. each of benzaldehyde and of benzyl alcohol per kilo of weight were injected subcutaneously into six rats, three animals being injected with each of the substances studied. Pooled 48 hour samples of urine were used for the isolation of the detoxication products by the procedure which was described above. The substances isolated on analysis proved to be identical with hippuric acid.

Experiments with dl-S-Benzylhomocysteine—2.0 gm. of *dl*-S-benzylhomocysteine were fed to each of the four rabbits and 1.0 gm. of the same substance was fed to each of the six rats. The substance was fed mixed with the food. The urine of each animal was then treated exactly as was described above. In every case N-acetyl-S-benzylhomocysteine was isolated from the urine, and typical analytical values are shown below.

	C	H	N	Acetyl	M.p. °C.
Rabbit. Found	58.33	6.45	5.30	16.00	129–130
Rat. "	58.33	6.45	5.17	16.21	129–130
Calculated for $C_{13}H_{17}NSO_3$	58.42	6.36	5.24	16.11	

N-Acetyl-S-benzylhomocysteine which was isolated from the urine was converted to S-benzylhomocysteine by the procedure which we used for the determination of the acetyl group (6). About 100 mg. of the substance were digested for 3 hours at 100° in 10 cc. of 25 per cent solution of *p*-toluenesulfonic acid. The digest was then cooled and the acidity was adjusted to the faint blue of Congo red with ammonia. On cooling in the refrigerator, crystalline material separated. It was centrifuged off, washed with cold water, and recrystallized several times from hot water. The dried material had a melting point of 239–247°, the nitrogen content was 5.98 per cent (micro-Dumas); calculated nitrogen percentage for S-benzylhomocysteine was 6.22. The isolated product was free from acetyl group.

As will be seen from the discussion, we had reason to believe that the isolated N-acetyl-S-benzylhomocysteine is probably of the levo form. We hope to present in a future communication a more detailed study of the transformation of the *d*-mercapturic acids into the *l* form *in vivo*. For this reason we have not included the data on the optical rotation of the isolated compounds in the present report.

Experiments with Benzyl Chloride—500 mg. of benzyl chloride were injected subcutaneously into each of the four rabbits, and 100 mg. of the same substance were similarly injected into each of six rats. The pooled urines of each group of animals were used for the isolation of benzylmercapturic acid by the procedure which was described above. The analytical values shown below indicate that the isolated product is identical with N-acetyl-S-benzylcysteine.

	C	H	N	Acetyl	M.p. °C.
Rabbit. Found	56.70	5.65	5.55	16.31	147–148
Rat. "	56.80	5.78	5.50	16.80	148–149
Calculated for $C_{12}H_{15}NSO_3$	56.92	5.93	5.54	16.98	

DISCUSSION

The results presented above indicate that neither benzyl alcohol nor benzaldehyde is formed from benzyl chloride in the animal body, inasmuch as both of these substances when administered to rats or rabbits yielded in the urine hippuric acid and not benzylmercapturic acid. The oxidation of benzaldehyde and its various derivatives to the corresponding benzoic acids in the animal body was observed previously by a number of workers (7). Our results offer evidence for the assumption that benzyl chloride was coupled directly with the —SH group of some substance in the animal body and after undergoing further metabolic changes was finally excreted in the urine as benzylmercapturic acid. The substance with which benzyl chloride combined was certainly not homocysteine, as the results presented above indicated. S-Benzylhomocysteine, when fed to rats or rabbits, yielded in the urine N-acetyl-S-benzylhomocysteine. Had benzyl chloride been coupled with homocysteine in the animal body, N-acetyl-S-benzylhomocysteine would have been excreted in the urine of animals after the administration of benzyl chloride. The results further show that S-benzylhomocysteine is not convertible into S-benzylcysteine in the rabbit or rat. These findings, however, do not preclude the possibility of conversion of homocysteine into cysteine in the animal body.

There is still the question as to whether the cysteine of benzylmercapturic acid is derived from the protein of the diet or of the tissues. Our earlier work on the synthesis of mercapturic acids in animals (8) left us almost convinced that the hydrocarbons which yield mercapturic acids in animals react with tissue protein first. The damaged tissue then undergoes repair, the efficiency in which is largely determined by the cystine and methionine of the diet. In view of the similarity in the detoxication of benzyl chloride and that of halogenated benzenes, naphthalene, and anthracene we are inclined to suggest that in the case of benzyl chloride, as in the case of the hydrocarbons, the tissue protein is attacked first.

Of particular interest in this connection is the observation that benzyl chloride induces sensitization in animals (9). In discussing the mechanism of sensitization of animals to simple organic substances, Heidelberger concludes that, "The simplest explanation at present would seem that in certain individuals conjugation of the drug or hapten takes place with the tissue protein, forming a new antigen which gives rise to the sensitization phenomenon" (10). From the above discussion it appears that the tissue protein may be the common factor in the mechanisms of sensitization and detoxication of benzyl chloride in animals.

As we pointed out previously (1), in the light of Knoop's theory regarding the rôle of the acetylation mechanism in the synthesis of amino acids *in vivo* (11) the synthesis of N-acetyl-S-benzylcysteine from S-benzylcysteine may indicate the formation of a corresponding derivative of α-keto-β-thiopropionic acid as the intermediate substance in the animal body. Similar considerations may apply to the synthesis *in vivo* of N-acetyl-S-benzylhomocysteine from S-benzylhomocysteine. In this case the corresponding derivative of α-keto-γ-thiobutyric acid may be the intermediate compound. If our deductions are correct, they could be demonstrated experimentally by the administration of *d*-S-benzylhomocysteine. If acetylation occurs in the manner described by Knoop (11) and du Vigneaud and Irish (12), *l*-N-acetyl-S-benzylhomocysteine would appear in the urine. Such a result would offer a direct demonstration of the convertibility of *d*-homocystine into the *l* enanthiomorph *in vivo*.

Grateful acknowledgment is made to Mr. J. Alicino for the microanalytical work.

SUMMARY

1. Benzyl chloride injected into rabbits and rats yielded in the urine N-acetyl-S-benzylcysteine.

2. Benzyl alcohol and benzaldehyde injected into rabbits and rats yielded in the urine hippuric acid. This indicates that neither benzyl alcohol nor benzaldehyde can be considered as an intermediate substance which is formed from benzyl chloride in the animal body.

3. *dl*-S-Benzylhomocysteine, when fed to rabbits and rats, yielded in the urine N-acetyl-S-benzylhomocysteine, the spatial configuration of which was not determined. Reasons are presented for the assumption that the isolated product might be the *l* form of the homocysteine derivative. The results further indicate that homocysteine is not involved directly in the detoxication of benzyl chloride, and that S-benzylhomocysteine is not convertible into S-benzylcysteine *in vivo*.

4. In view of the similarity in the detoxication of benzyl chloride, halogenated benzenes, and certain hydrocarbons it is assumed that benzyl chloride, when administered to animals, reacts with tissue protein as the first step in the course of its detoxication to yield benzylmercapturic acid.

5. A possible connection between the mechanism of sensitization to benzyl chloride in animals and that of detoxication of this substance is pointed out.

BIBLIOGRAPHY

1. Stekol, J. A., *J. Biol. Chem.*, **124**, 129 (1938).
2. du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933).
3. White, A., and Beach, E. F., *J. Biol. Chem.*, **122**, 219 (1937-38).
4. du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.*, **109**, 97 (1935).
5. Butz, L. W., and du Vigneaud, V., *J. Biol. Chem.*, **99**, 135 (1932-33).
6. Elek, A., and Harte, R. A., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 267 (1936).
7. Sieber, N., and Smirnow, A., *Monatsch. Chem.*, **8**, 88 (1887). Cohn, R., *Z. physiol. Chem.*, **17**, 274 (1893). Roehmann, F., Biochemie, Berlin, 413 (1908). Sherwin, C. P., and Hynes, W. A., *J. Biol. Chem.*, **47**, 297 (1921).
8. Stekol, J. A., *J. Biol. Chem.*, **117**, 147 (1937); **118**, 155 (1937); **122**, 333 (1937-38); *Proc. Soc. Exp. Biol. and Med.*, **35**, 623 (1937).
9. Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, **64**, 625 (1936).
10. Heidelberger, M., in Schmidt, C. L. A., The chemistry of the proteins and amino acids, Springfield, 972 (1938).
11. Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, **146**, 267 (1925).
12. du Vigneaud, V., and Irish, O. J., *J. Biol. Chem.*, **122**, 349 (1937-38).

A STUDY OF THE UTILIZATION OF THE OPTICAL ISOMERS OF N,N'-DIMETHYLCYSTINE

BY MARIAN WOOD KIES, HELEN M. DYER, JOHN L. WOOD,
AND VINCENT DU VIGNEAUD

(From the Departments of Biochemistry, School of Medicine, George Washington University, Washington, and Cornell University Medical College, New York City)

(Received for publication, January 18, 1939)

In instances in which the unnatural form of an amino acid is capable of supporting the growth of animals on diets devoid of, or deficient in, the naturally occurring form, it is rather generally accepted that the animal is able to convert the unnatural isomer into its antipode through the keto acid (1–4). However, when the unnatural isomer cannot be so utilized, such as in the case of cystine (5), lysine (6–8), and certain other amino acids (3), it may be questioned whether oxidation to the keto acid occurs before some irreversible reaction involving another part of the molecule takes place. In these instances, the keto acid corresponding to the amino acid would not be available, therefore, for the asymmetric synthesis of the latter. On the other hand, if oxidative deamination does occur first, it may be that the keto acid is so unstable that opportunity for conversion to the natural form is not afforded. Such possibilities were pointed out in connection with our discussion of the oxidizability of the stereoisomers of cystine (9).

We have therefore become interested in ascertaining whether the keto acid of cystine can be utilized for growth. Since the keto acid is not yet available, an indirect attack based on circumstantial evidence would seem to be justified. The present communication is based on such an approach.

As discussed in a previous communication (10), there is considerable reason to suspect that N-methylamino acids which are able to support growth in lieu of the natural amino acid do so by virtue of the fact that they are deaminized oxidatively to the corresponding keto acids and that the latter are converted to the amino acids. It has already been shown that amino-N-methyltryptophane (11), amino-N-methylhistidine (12), N,N′-dimethylhomocystine (10), and N-methylmethionine (10) can be utilized by the body for growth purposes. In our work with the latter two amino acids, arguments were presented that demonstration of growth-promoting power of an N-methylamino acid was tantamount to demonstrating that the corresponding keto acid was likewise capable of being utilized. It is realized, of course, that this conclusion is based on inference, but we feel it is justified at our present state of knowledge.

On the basis of the preceding considerations, we felt that it would be of interest to study the utilization of the N-methyl derivative of cystine. Both optically active forms were studied. It is of interest in this connection that Gordon (13) found *l*(+)-amino-N-methyltryptophane (14) to be utilizable but that the *d*(−) isomer was not.

The N,N′-dimethylcystine was not available at the time this study was contemplated. Previous work had shown that the usual method of synthesis of N-methylamino acids involving methylation of the benzenesulfonyl derivative did not lead to the preparation of the compound. Acid hydrolysis of the acyl derivative resulted in the cleavage of the nitrogen from the carbon skeleton of the cystine moiety (15). The observation by du Vigneaud and Behrens (16), that the toluenesulfonyl group could be removed from an amino acid derivative by reduction with sodium in liquid ammonia, opened a new approach to the synthesis of N,N′-dimethylcystine. The methylated toluenesulfonyl derivative of *l*-cystine was therefore prepared and was treated with sodium in liquid ammonia. The reduction procedure was found to be effective, since dimethyl-*l*-cystine could be isolated from the reaction mixture, after removal of the ammonia and subsequent oxidation of its aqueous solution with air. The product so obtained was partially racemized. Further work showed it advantageous both from the standpoint of yield and purity to isolate the S-benzyl-N-methyl-*l*-cysteine as an intermediary step after the detosylation before preparing the dimethyl-*l*-cystine. The benzyl derivative was formed by adding benzyl chloride to the liquid ammonia reaction mixture. The benzyl derivative after isolation and recrystallization was then treated with sodium in liquid ammonia and the N-methyl-*l*-cysteine so formed was oxidized to the disulfide by the addition of an ethereal solution of iodine to the liquid ammonia reduction mixture. The N,N′-dimethyl-*d*-cystine was prepared from *d*-cystine obtained from the resolution of *dl*-cystine (17).

The properties of the N,N′-dimethyl-*l*-cystine which we prepared and used in the feeding work agree with those recently reported for the compound by Bloch and Clarke (18) who prepared the compound by a somewhat different procedure which was based, however, on the reduction of the toluenesulfonyl derivative with sodium in liquid ammonia.

EXPERIMENTAL

Preparation of d- and l-Di-p-Toluenesulfonylcystine—A solution of 13.5 gm. of *l*-cystine in 114 cc. of N NaOH was stirred vigorously with a mechanical stirrer and a solution of 42 gm. of *p*-toluenesulfonyl chloride in 100 cc. of ether was added in ten portions at 15 minute intervals. Each portion was preceded by the addition of 11.4 cc. of 2 N NaOH. Additional ether was added from time to time to dissolve some acid chloride which had precipitated. A brown color gradually developed. When the addition of the sulfonyl chloride was complete, the solution was stirred for 30 minutes longer. After removal of the ether layer the solution was extracted with 100 cc. of ether. The dissolved ether was then removed by warming, and concentrated HCl was slowly added to the warm solution with vigorous shaking. The crystals which precipitated first were quite colorless but upon further addition of the HCl the precipitate began to bring down some of the colored impurity. At this stage it was found best to treat the mother liquor with norit and then to proceed with the addition of HCl. Treatment of the more alkaline solution itself with norit failed to remove the color. 22 gm. of di-*p*-toluenesulfonylcystine were obtained, which represented 74 per cent of the theoretical yield. The product gave a negative Sullivan test for cystine. After recrystallization by solution in NH_4OH and precipitation with HCl, the compound melted at 214°[1] and had a rotation of $[\alpha]_D^{22} = -153°$ for a 1 per cent solution in water containing an equivalent amount of NH_4OH. The melting point agreed with that given by Bloch and Clarke (18). As pointed out by the latter authors, McChesney and Swann (19) reported a melting point of 201–203° for the compound recrystallized from 60 per cent alcohol.

---

[1] All melting points reported in this paper are corrected.

Preparation of d- and l-S-Benzyl-N-Methylcysteine—To 5 gm. of di-*p*-toluenesulfonyl-*l*-cystine dissolved in 55 cc. of N NaOH in a 200 cc. round bottomed flask, 2.3 cc. of methyl iodide were added. The stopper was wired to the flask and the solution was warmed to about 70°. The flask was shaken vigorously until the methyl iodide layer had disappeared.

After the solution had cooled, it was extracted with ether. The aqueous layer was then overlaid with 100 cc. of ether and was strongly acidified with HCl. The aqueous layer was reextracted with small portions of ether until it gave a negative test for disulfide. The combined ether extracts were then washed with water containing a small amount of sodium bisulfite and were distilled. The amorphous residue which was left was covered with 200 cc. of liquid ammonia and about 3 gm. of sodium were added in small pieces while the solution was stirred mechanically. The color of the solution changed from colorless to red at the end of the addition of 8 moles of sodium. A very slow reaction then took place with the remaining sodium and, at the end of the addition of 16 moles, the solution was very thick and a permanent blue color remained. The excess sodium was destroyed with ammonium chloride and 2.3 cc. of benzyl chloride were slowly added. The liquid ammonia was allowed to evaporate and 50 cc. of ice and water were added. The resulting solution was extracted twice with ether and then acidified with HCl to pH 6. The precipitated crystals of S-benzyl-N-methyl-*l*-cysteine were filtered and washed successively with water, a slight amount of alcohol, and then with ether. The product weighed 2.2 gm., which represented 53 per cent of the theoretical amount. After recrystallization from hot dilute HCl, the compound possessed a rotation of $[\alpha]_D = +64.5°$ for a 1 per cent solution in N HCl and a melting point of 207–208° (with decomposition).

$C_{11}H_{15}O_2NS$. Calculated, N 6.4; found, N 6.2

The *d* isomer was prepared in a similar manner. It possessed a rotation of $[\alpha]_D = -65°$ and a melting point of 207–208° (with decomposition).

$C_{11}H_{15}O_2NS$. Calculated, N 6.4; found, N 6.2

Preparation of d- and l-N,N'-Dimethylcystine—6 gm. of S-benzyl-N-methyl-*l*-cysteine were dissolved in 200 cc. of liquid ammonia and about 3.6 gm. of sodium were added in small pieces until the blue color of excess sodium was permanent. Ammonium iodide was added to remove the excess sodium and then an equivalent amount of iodine dissolved in dry ether was slowly added. The ammonia was allowed to evaporate and the residue was taken up in about 20 cc. of ice and water. The ice-cold solution was extracted with ether. The pH was adjusted to 6 with HI and 4 to 5 volumes of absolute alcohol were added. The crystalline mass which precipitated was centrifuged and was washed with absolute alcohol. The crystals were then suspended in a small amount of water and were filtered. A second crop was obtained by adding alcohol to this aqueous filtrate. 2.3 gm. of N,N'-dimethyl-*l*-cystine were obtained, which represented 65 per cent of the theoretical yield. The material was free of cystine, as shown by the Sullivan test. The compound was recrystallized by dissolving a portion in a minimum amount of N NaOH and adding HI until the solution was just acid to litmus. After an hour, fine needles separated. These were washed with cold water and with alcohol. They melted with decomposition at 216–218° and had a rotation of $[\alpha]_D^{22} = +75.5°$ for a 1 per cent solution in N HCl.

$C_8H_{16}O_4N_2S_2$. Calculated, S 23.9, N 10.4; found, S 23.7, N 10.5

The *d* isomer was likewise prepared. It possessed a rotation of $[\alpha]_D^{26.5} = -75°$ and melted at the same temperature as its optical antipode.

$C_8H_{16}O_4N_2S_2$. Calculated, N 10.4; found, N 10.1

Growth Studies—Two litters of rats were used to test N,N'-dimethyl-*l*-cystine and N,N'-dimethyl-*d*-cystine for their availability for growth. The basal diet was identical with that used in our previous studies in which a cystine-deficient diet was de-

TABLE I

Food Consumption of Litter 1

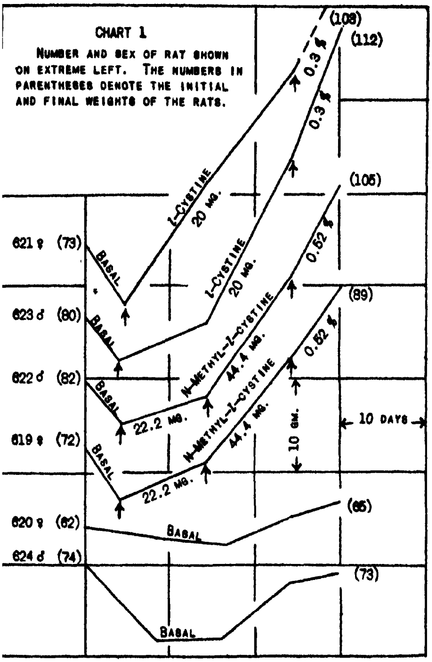
Rat No. and sex	Days	Daily supplement to basal diet	Daily food consumption
			gm.
620 ♀	1–30		6.2
624 ♂	1–30		5.8
621 ♀	1– 4		5.5
	4–24	20 mg. *l*-cystine	7.6
	24–30	0.3% "	8.1
623 ♂	1– 4		6.8
	4–24	20 mg. *l*-cystine	7.8
	24–30	0.3% "	7.8
619 ♀	1– 4		4.8
	4–14	22 mg. N-methyl-*l*-cystine (2 pills)	4.9
	14–24	44 " " (6 ")	7.6
	24–30	0.52% "	8.1
622 ♂	1– 4		6.8
	4–14	22 mg. N-methyl-*l*-cystine (2 pills)	5.5
	14–24	44 " " (6 ")	9.3
	24–30	0.52% "	8.1

TABLE II

Food Consumption of Litter 2

Rat No. and sex	Days	Daily supplement to basal diet	Daily food consumption
		per cent	*gm.*
687 ♂	1–24		3.7
688 ♀	1–24		4.7
685 ♂	1– 4		5.0
	4–24	0.45 N-methyl-*d*-cystine	4.1
686 ♀	1– 4		4.5
	4–24	0.45 N-methyl-*d*-cystine	4.4
683 ♀	1– 4		5.0
	4–22	0.45 N-methyl-*l*-cystine	5.2
684 ♂	1– 4		6.0
	4–22	0.45 N-methyl-*l*-cystine	6.4
681 ♀	1– 4		4.5
	4–24	0.30 *l*-cystine	4.9
682 ♂	1– 4		4.3
	4–24	0.30 *l*-cystine	4.9

sired (10), having the following percentage composition: casein 6.0, dextrin 33.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Osborne and Mendel (20)) 4.0, agar 2.0, and milk vitamin concentrate (21) 16.0. It was fed *ad libitum* and a record was kept of the food consumption, which is given in Tables I and II.



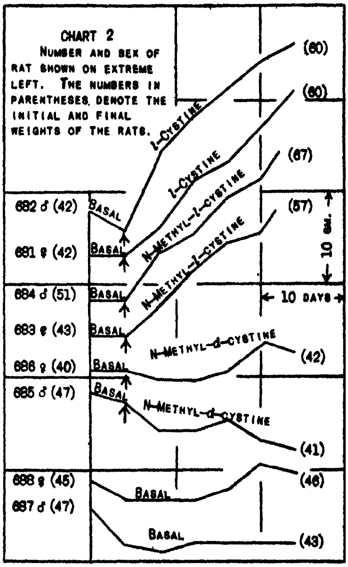


CHART 1. Litter 1. Growth curves of rats on a cystine-deficient diet supplemented with *l*-cystine and N,N′-dimethyl-*l*-cystine.

CHART 2. Litter 2. Growth curves of rats on a cystine-deficient diet, supplemented with *l*-cystine, N,N′-dimethyl-*l*-cystine, and N,N′-dimethyl-*d*-cystine.

The animals were given the basal diet for a preliminary period of 4 days. At the end of this period Rats 619 and 622 of Litter 1 were given dimethyl-*l*-cystine daily in two dextrin pills, each pill containing 11.1 mg. of the cystine derivative. After 10 days, the daily amount was increased to 44.4 mg. to see whether better growth would result. The dosage was furthermore divided into six pills daily to afford the opportunity for better utilization. Rats 621 and 623 of Litter 1 served as controls, receiving 20 mg. of *l*-cystine daily as a supplement to the basal diet. As in the case of dimethyl-*l*-cystine, for the first 10 days the supplement was divided into two daily doses and thereafter into six. For the last 6 days of the experimental period, the dimethyl-*l*-cystine and *l*-cystine were incorporated in the diet at a concentration of 0.52 per cent and 0.3 per cent respectively. Rats 620 and 624 were kept on the basal diet throughout the experimental period.

In planning the experiment with Litter 2, it was decided to incorporate all of the supplements in the diet instead of giving them in the form of pills. There were some indications from the results with Litter 1 that better growth resulted when the dimethyl-*l*-cystine was made available to the body in small amounts repeatedly during the day. The dimethylcystine derivatives were administered in the diet at a somewhat higher level than the *l*-cystine. Rats 683 and 684 were given the dimethyl-*l*-cystine and Rats 685 and 686 the dimethyl-*d*-cystine at a concentration of 0.45 per cent in the diet, equivalent to 0.4 per cent *l*-cystine. Rats 681 and 682 received *l*-cystine at a concentration of 0.3 per cent and Rats 687 and 688 were kept on the basal diet throughout the experimental period.

As the individual growth curves in Charts 1 and 2 demonstrate, the animals were able to grow with N,N′-dimethyl-*l*-cystine in lieu of *l*-cystine. On the other hand there was little difference, if any, between the behavior of the animals on the basal diet and those which had received the dimethyl-*d*-cystine as a supplement.

DISCUSSION

As previously discussed in the introduction to this paper, there is evidence to believe that in the utilization for growth purposes of N-methylamino acids there occurs an oxidative deamination to the keto acid followed by the synthesis of the amino acid. The ability of dimethyl-*l*-cystine to support the growth of animals on a cystine-deficient diet, as shown in the present experiments, might be explained on this basis; that is, the dimethyl-*l*-cystine would be oxidatively deaminized to dithiodipyruvic acid and the latter converted to *l*-cystine. The positive growth results with dimethyl-*l*-cystine would therefore lead to the inference that the body is capable of converting dithiodipyruvic acid into cystine and that this keto acid should support growth under the conditions of these experiments.

In the case of leucine and isoleucine, the keto acid analogues have actually been prepared and studied (3). These keto acids have been found to support growth on diets devoid of the corresponding amino acids. This is of particular significance to the present discussion because both *d*-leucine and *d*-isoleucine are incapable of supporting growth. It will be seen that the behavior of *d*-leucine and *d*-isoleucine and their keto acids is quite parallel to the inferred behavior of the dithiodipyruvic acid and to the known behavior of *d*-cystine.

Our finding that dimethyl-*d*-cystine is incapable of being utilized, although the dimethyl-*l*-cystine can be, is of interest in the light of the observations of Gordon that the *d*(−)-amino-N-methyltryptophane cannot be utilized although the *l*(+) compound could be. It would begin to appear that methylation of the amino acid corresponding to the unnaturally occurring series prevents oxidative deamination. In the case of tryptophane the effect of methylation is perhaps more striking, since *d*-tryptophane itself can be utilized.

SUMMARY

The N,N′-dimethyl-*l*-cystine is capable of supporting the growth of animals in lieu of *l*-cystine, whereas the N,N′-dimethyl-*d*-cystine gave no indication of its being utilized in this manner.

It has been inferred from these data that the keto acid of cystine should be capable of supporting growth under the experimental conditions employed in this work.

BIBLIOGRAPHY

1. Berg, C. P., and Potgieter, M., *J. Biol. Chem.*, **94**, 661 (1931–32).
2. du Vigneaud, V., Sealock, R. R., and Van Etten, C., *J. Biol. Chem.*, **98**, 565 (1932).
3. Rose, W. C., *Physiol. Rev.*, **18**, 109 (1938).
4. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1937–38).
5. Loring, H. S., Dorfman, R., and du Vigneaud, V., *J. Biol. Chem.*, **103**, 399 (1933).
6. McGinty, D. A., Lewis, H. B., and Marvel, C. S., *J. Biol. Chem.*, **62**, 75 (1924–25).

7. Berg, C. P., and Dalton, J. L., *Proc. Soc. Exp. Biol. and Med.*, **31,** 709 (1933–34).
8. Berg, C. P., *J. Nutrition*, **12,** 671 (1936).
9. du Vigneaud, V., Craft, H. A., and Loring, H. S., *J. Biol. Chem.*, **104,** 81 (1934).
10. Patterson, W. I., Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **116,** 277 (1936).
11. Gordon, W. G., and Jackson, R. W., *J. Biol. Chem.*, **110,** 151 (1935).
12. Fishman, J. B., and White, A., *J. Biol. Chem.*, **113,** 175 (1936).
13. Gordon, W. G., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **123,** p. xliii (1938).
14. Cahill, W. M., and Jackson, R. W., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **123,** p. xviii (1938).
15. Fruton, J. S., and Clarke, H. T., *J. Biol. Chem.*, **106,** 667 (1934).
16. du Vigneaud, V., and Behrens, O. K., *J. Biol. Chem.*, **117,** 27 (1937).
17. du Vigneaud, V., Dorfman, R., and Loring, H. S., *J. Biol. Chem.*, **98,** 577 (1932).
18. Bloch, K., and Clarke, H. T., *J. Biol. Chem.*, **125,** 275 (1938).
19. McChesney, E. W., and Swann, W. K., *J. Am. Chem. Soc.*, **59,** 1116 (1937).
20. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37,** 572 (1919).
21. Supplee, G. C., Kahlenberg, O. J., and Flanigan, G. E., *J. Biol. Chem.*, **93,** 705 (1931).

A NEW PRINCIPLE FOR THE DETERMINATION OF AMINO ACIDS, AND ITS APPLICATION TO COLLAGEN AND GELATIN

By MAX BERGMANN and WILLIAM H. STEIN

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, January 17, 1939)

The fundamental task of protein chemistry is the establishment of the composition of the various proteins in terms of the constituent amino acids in the same manner as the composition of simpler molecules has been ascertained in terms of their constituent atoms. We have attempted, therefore, to develop a general principle that would permit the determination of amino acids to be made with a precision approaching that attained in the elementary analysis of simpler molecules. The present communication contains a description of this new principle, and of its application to the determination of glycine, *l*-alanine, *l*-leucine, and *l*-proline in solutions of known content. In addition, determinations of *l*-proline and glycine in hydrolysates of cattle tendon collagen and of commercial gelatin preparations are described.

Analytical Procedure

All the methods hitherto described for the determination of amino acids have one feature in common. In each instance an attempt is made to isolate as much as possible of a given amino acid—either as such or as a salt, a derivative, or another transformation product. With respect to the precipitation of amino acids as salts, all efforts have been directed towards the discovery of reagents permitting the quantitative removal of an amino acid from protein hydrolysates. The well-nigh insurmountable difficulties attending the development of a reagent which will selectively and quantitatively remove any amino acid from so complex a mixture are evidenced by the fact that no reagent fulfilling these requirements has as yet been found. It has frequently been suggested that the accuracy of determinations involving salt precipitations could be improved by incorporating in the final result a correction which allows for the solubility of the salt in the mother liquor. The fact has been neglected, however, that the solubility of an amino acid salt in its mother liquor is a rather complex function of the composition of this mother liquor. Without any knowledge of the nature of this function, it is impossible to ascertain how much of an amino acid salt has, under given conditions, escaped precipitation. In consequence, there is a varying element of doubt attached to all the figures for the amino acid content of a protein which have been obtained with the aid of methods employing salt precipitations.

The experiments reported in the present paper were undertaken to study the solubility behavior of amino acid salts. These studies resulted in a method for the determination of amino acids which foregoes any attempt at a complete precipitation of an amino acid as a salt. On the contrary, in the new method only those amino acid salts that possess a measurable solubility can be employed (1).

This method is founded upon our experimental observation that the amount of an amino acid salt precipitated from a solution is, at equilibrium, a function of the concentration of its ions in solution. The general case under investigation is that in which an amino acid A combines with an acidic reagent RH to form the binary salt AH·R. It was found that the quantity of the salt AH·R, which crystallizes from a solution containing the ions AH^+ and R^-, is such that at the completion of the precipitation the product of the concentrations of the ions AH^+ and R^- remaining in solution has assumed a value that is a constant for a given solution at a given temperature.

$$[AH^+] \times [R^-] = \text{constant} \qquad (1)$$

In order to discuss the applicability of Equation 1 to the determination of an amino acid, the following example may be considered. To a solution containing Y moles of an amino acid in a volume of v ml. are added r' moles of reagent. After equilibrium has been attained, the precipitated amino acid salt is filtered, weighed, analyzed, and thus found to contain x_1 moles of the reagent. The difference $(r' - x_1)$ is the quantity of reagent remaining in solution. This quantity, when reduced to a 1 liter basis, gives the concentration of the reagent remaining in solution. The concentration of A remaining in solution may also be found from the weight and composition of the precipitate, for the precipitate contains equimolar amounts x_1 of reagent and y_1 of amino acid. Therefore $Y - y_1$ is the amount of A remaining in solution. This quantity must also be reduced to a 1 liter basis in order to obtain the concentration of the amino acid remaining in solution.

If one now employs the concentrations of amino acid A and reagent RH remaining in solution, instead of the concentrations of the respective ions AH^+ and R^-, Equation 1 may be written in the following form.[1]

$$\frac{1000(r' - x_1)}{v} \times \frac{1000(Y - y_1)}{v} = C \qquad (1, a)$$

The constant, C, and Y are the only unknowns in Equation 1, *a*. In order to eliminate the constant, it is necessary only to perform a second experiment in which an identical sample of amino acid solution is employed, but a larger amount of reagent r''. A larger amount of precipitate is formed in this experiment, but it again contains equivalent molar quantities x_2 of reagent and y_2 of amino acid. This second experiment can be represented by Equation 1, *b*.

$$\frac{1000(r'' - x_2)}{v} \times \frac{1000(Y - y_2)}{v} = C \qquad (1, b)$$

A combination of the Equations 1, *a* and 1, *b* gives Equation 2.

$$(r' - x_1)\ (Y - y_1) = (r'' - x_2)\ (Y - y_2) \qquad (2)$$

From this equation the value for Y, the amino acid content of the sample originally taken, may readily be calculated.

Fig. 1 illustrates an experiment in which r' and r'' moles of a reagent are added to two equal samples of a solution, each containing Y moles of amino acid. The moles of reagent added are plotted against the moles of amino acid present. It is apparent that when the precipitation has ceased, x' moles of reagent and y' moles of amino acid remain in the solution to which r' moles of reagent were added. Similarly, x'' moles of reagent and y'' moles of amino acid remain in the solution to which r'' moles of reagent were added. Thus we find that the course of the precipitation with increasing amounts of reagent follows a hyperbolic curve passing through the points C' and C''.

---

[1] It will be noticed that in substituting the concentrations of A and RH for the concentrations of AH^+ and R^-, the activity coefficients of these ions are neglected. The assumption is made that the activities, whatever they may actually be, remain nearly constant over the concentration range covered by the analytical precipitations. The admissibility of this assumption is supported by the experimental data reported in Table I.

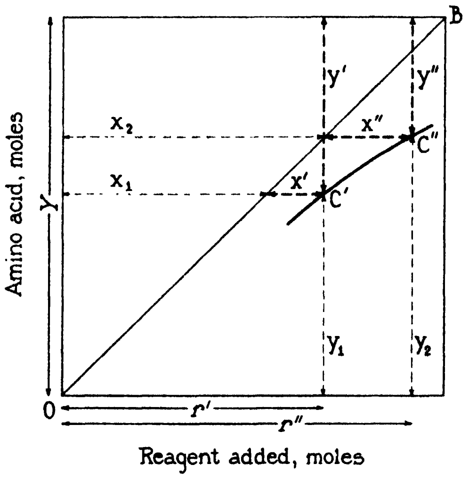


FIG. 1. Determination of amino acids by means of the solubility product. The symbols have the values described in the text.

From Fig. 1 it follows that Equation 2 may also be written

$$x' \times y' = x'' \times y'' = C \tag{2, a}$$

This constant, reduced to a 1 liter basis, is usually referred to as the solubility product of the salt. Since the new analytical method is based upon the law of the constancy of the solubility product, it may, for the sake of brevity, be designated the solubility method.

In Table I are reported the experimental data from a number of determinations—made with the solubility method—of amino acids in solutions of known content.

TABLE I*

Amino Acid Analyses by Solubility Method

Amino acid	Amino acid content employed	Reagent employed	Amino acid pptd.	Reagent left in solution	Amino acid content found	Solubility product K
	mole	*mole*	*mole*	*mole*	*mole*	
l-Proline	0.00420 in 205 cc. 0.075 N HCl + 135 cc. methanol	0.00427†	0.00323	0.00104		8.72×10^{-6}
		0.00504	0.00353	0.00151	0.00419	8.80×10^{-6}
l-Alanine	0.00299 in 15 cc. N HCl	0.00188‡	0.00157	0.00031		1.96×10^{-3}
		0.00282	0.00223	0.00059	0.00300	1.99×10^{-3}
"	0.00300 in 15 cc. N HCl	0.00187‡	0.00151	0.00036		2.39×10^{-3}
		0.00281	0.00217	0.00064	0.00302	2.36×10^{-3}
l-Proline	0.0150 in 75 cc. N HCl	0.00936‡	0.00758	0.00178		2.34×10^{-3}
		0.01405	0.01084	0.00321	0.0149	2.37×10^{-3}
l-Leucine	" "	0.00936‡	0.00885	0.00051		5.55×10^{-4}
		0.01405	0.01274	0.00131	0.0152	5.24×10^{-4}
Glycine	" "	0.00585‡	0.00365	0.00220		4.42×10^{-3}
		0.01405	0.00954	0.00451	0.0152	4.36×10^{-3}
l-Proline + glycine	0.0150 + 0.00500 in 75 cc. N HCl	0.00936‡	0.00771	0.00165		2.14×10^{-3}§
		0.01405	0.01111	0.00294	0.0154	2.03×10^{-3}§
l-Leucine + *l*-proline	0.00790 + 0.00381 in 75 cc. 0.91 N HCl	0.00468‡	0.00394	0.00074		5.18×10^{-4}‖
		0.00707	0.00576	0.00130	0.00817	4.92×10^{-4}‖

---

* We should like to state that we attach no great significance to the absolute values of the solubility products reported in the last column. The complex radicals used as precipitating agents undergo a slow decomposition. In addition, the various determinations were made under slightly different experimental conditions, exerting varying influences upon the solubility products. From the analytical point of view it is important, however, that the two simultaneous precipitations of each determination were performed under identical experimental conditions and consequently resulted in identical solubility products. For example, the two alanine determinations in Table I differ from each other in the absolute values of their solubility products. However, the solubility product is a constant for the two simultaneous precipitations of each determination. Moreover, the alanine values obtained in the two independent determinations are in good agreement with each other and with the amount of alanine actually present.

† Ammonium rhodanilate.

‡ Sodium dioxpyridate.

§ For proline.

‖ For leucine.

The high degree of accuracy achieved in the determinations reported in Table I is indeed gratifying and may, in part, be a consequence of the paucity and simplicity of the manipulations required in each determination. In applying the solubility method to protein hydrolysates, it is essential to preserve this simplicity in so far as possible. To this end it is advisable to perform each precipitation directly upon a fresh sample of hydrolysate, all manipulative procedures which might entail losses being avoided.

The solubility method should have a wide application in the determination of amino acids, for there are many reagents which form crystalline salts with amino acids; some of these reagents will be described in succeeding papers of this series. At present it may be pointed out that the solubility method is by no means restricted to the determination of amino acids, but may be applied also to the determination of a variety of constituents of complex mixtures.

One of the advantages of the solubility method is the relative ease with which the accuracy of the results obtained by it can be checked. There are several checks that can be applied. In the first place, when one is dealing with solutions of unknown amino acid content, it is advisable to perform three or four precipitations (rather than only two) with equal samples of the amino acid solution but increasing amounts of reagent. From a series of three precipitations, three values for the amino acid content of a solution may be calculated; while from a series of four precipitations, six values may be calculated. None of the values should be accepted unless all of them are in good agreement. In the second place, the determination may be repeated with a different concentration of the amino acid. The results of the determinations performed at different concentrations should check one another. In the third place, a known quantity of the amino acid under investigation may be added to the analytical solution before the precipitations are performed. The result of the determination, reduced by the amount of amino acid added, should closely approximate the result obtained when no amino acid is added. The description of the application of these checks to the determination of *l*-proline in protein hydrolysates will be found in the experimental section of this paper.

In the application of the solubility method two types of reagents are available. One type comprises those reagents which are specific for a single amino acid and do not precipitate other amino acids under ordinary conditions. Rhodanilic acid, when used as its ammonium salt, seems to represent this type of reagent, inasmuch as it precipitates only proline (2). A second, and more general type of reagent, forms salts with a variety of amino acids. These salts, however, frequently exhibit differences in the values of their solubility products (Table I). Since the relative concentrations of amino acids in the hydrolysates of different proteins vary within wide limits, the same reagent may be used for the determination of one amino acid in one hydrolysate and of another amino acid in another hydrolysate. In order to illustrate this procedure in an artificial mixture, there are described in this paper determinations, made with dioxpyridic acid (3), of *l*-leucine in a mixture of *l*-leucine and *l*-proline, and of *l*-proline in a mixture of *l*-proline and glycine.

When a reagent of the second type is applied to the determination of any component of a mixture of amino acids of unknown content, either or both of the following procedures may be employed to insure the precipitation of a single amino acid salt. The mixture may be diluted to a point at which the solubility product of only one component of the mixture with the reagent is surpassed. At such a dilution only a single amino acid salt is precipitated. Sometimes, however, the various amino acids are present in such relative amounts that there can be found no dilution at which only one amino acid is precipitated by a given reagent. This difficulty may be overcome by the addition of a known quantity of the amino acid under determination. These procedures will be exemplified in forthcoming papers on the composition of proteins.

Protein hydrolysates sometimes contain several stereoisomeric forms of an amino acid. If both the *l* and the *d* forms of an amino acid are present, they will behave, in the course of a determination by the solubility method, as two amino acids, the salts of which, with a symmetrical reagent, possess identical solubility products. If there is a preponderant quantity of one of the stereoisomers, the determination may be performed at such a dilution that only the predominating isomer is precipitated. If, on the other hand, both the *l* and the racemic form of an amino acid are present in solution, they may behave as two different amino acids, the salts of which possess different solubility products.

The accuracy of the solubility method so far surpasses that of the methods hitherto available that it seems worth while to apply it to a reinvestigation of the amino acid content of many proteins. Thus we intend to subject the periodicity theory of protein structure to a series of rigorous checks.[2]

Determinations of l-Proline and Glycine in Gelatin and Collagen

In order to test the applicability of the solubility method to protein hydrolysates, *l*-proline was determined in hydrolysates of gelatin and of collagen of cattle Achilles tendon. For a comparison the glycine content of these proteins was reinvestigated. Glycine was precipitated by means of potassium trioxalatochromiate, according to the procedure recently described (7). The results of these glycine determinations are reported in Table II, while the experimental details will be found in the experimental section of this paper.

The agreement among these values is rather striking. It indicates that the glycine determination, by means of potassium trioxalatochromiate, is sufficiently precise to permit a comparative study of the collagens of different tissues and of various species. In this respect it seems of interest that the glycine content of the collagen and the gelatins investigated is the same.

The average glycine content, 26.5 per cent, found for several gelatins in these experiments is only slightly higher than the 25.5 per cent found by Dakin (8).

---

[2] Doubts as to the validity of the periodicity theory have recently been expressed by Astbury (4). After discussing the stoichiometry of the keratin molecule on the basis of the available constituent analyses of sheep wool, he emphasizes that frequencies such as eight and nine cannot coexist within the same protein molecule. As a matter of fact, the coexistence of these frequencies has never been claimed. Moreover, we feel that constituent analyses of protein mixtures of varying composition, such as the varieties of sheep wool, should not be employed as a basis for the calculation of amino acid ratios within the keratin molecule. Still less acceptable is the calculation, by Astbury, of the leucine ratio within the keratin molecule on the basis of a leucine determination performed with sheep wool in 1907 by Abderhalden and Voitinovici (5) by means of the ester method; *i.e.*, by a method known to give incomplete yields of a mixture of leucine, isoleucine, and valine (6).

With the figure of 26.5 per cent glycine, one may easily calculate that 100 gm. of water and ash-free gelatin or collagen would yield 0.353 gm. equivalent of glycine. The average weight of all the known amino acid residues in gelatin is about 89.2. This value is, in all probability, too low, inasmuch as the amino acid residues yet to be determined in gelatin may possess a higher average residue weight. We suggest an average residue weight of about 125 as a fair estimate for these undetermined residues. If these residues are taken into account, the corrected average residue weight for the total number of amino acid residues in gelatin would be 93.7. 100 gm. of gelatin therefore should yield on complete hydrolysis 1.067 gm. equivalents of the average amino acid. If one out of every three amino acids in gelatin were glycine, then 100 gm. of the protein should yield 0.356 gm. equivalent of glycine or 26.7 per cent.[3] The values found are in excellent agreement with this calculated figure. It appears therefore that one out of every three amino acid residues in gelatin and in collagen is indeed glycine, as has already been suggested by Astbury (9) on the basis of Dakin's glycine estimation (8).

TABLE II

Glycine Content of Several Protein Hydrolysates

Protein	Amount of protein employed	Amount of glycine added	Total N of ppt.	NH_3-N of ppt.	Glycine found	Glycine in protein
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Pig skin gelatin	1.81		82.6	1.8	471	26.0
	0.905	230	81.5	1.4	466	26.1
Coignet Gold Label gelatin	1.72		81.2	1.3	465	27.0
	0.860	230	80.0	0.9	461	26.9
Cattle Achilles tendon collagen	1.56		72.1	1.9	409	26.2
	0.780	230	76.5	1.3	438	26.7

By means of the solubility method, proline was determined as rhodanilate by precipitation with ammonium rhodanilate. The method of procedure will be found in the experimental section.

---

[3] If the average residue weight of the undetermined amino acids in gelatin were 130 instead of 125, then 100 gm. of gelatin should yield 0.355 gm. equivalent of glycine or 26.6 per cent.

The values obtained are reported in Table III. The average of the proline estimations in two commercial gelatins and in collagen of cattle Achilles tendon is 17.5. The single determinations do not deviate from the average by more than 2.5 per cent of the final value.

In an earlier paper from this laboratory, the value 19.7 per cent was reported for the proline content of gelatin. Our earlier value was obtained from experiments in which we endeavored to precipitate proline as completely as possible and to apply an empirical correction. As is pointed out in the present communication, values obtained by a "maximum precipitation" method can never be thoroughly accurate.

TABLE III

Proline Content of Several Protein Hydrolysates

Protein	Amount of protein employed	Total volume	Proline added	Proline found in protein	No. of precipitations
	gm.	*cc.*	*gm.*	*per cent*	
Pig skin gelatin	2.53	330		17.5	3
	2.48	450		17.6	3
	1.27	330	0.249	17.7	3
Label	2.59	330		17.0	3
gelatin	2.59	330		17.6*	3
	1.21	330	0.250	17.4	2
tendon	2.33	330		17.1	3
collagen	1.09	330	0.251	17.8	3

* This determination was performed by Dr. H. R. Ing in this laboratory.

On the other hand, there is as yet no proof that the value 17.5 per cent, obtained for *l*-proline by the solubility method, represents the total proline content of the original gelatin and collagen. It seems possible that a part of the proline is racemized during the hydrolysis of the protein with boiling hydrochloric acid. The extent of racemization during hydrolysis is at present being thoroughly investigated in this laboratory. In the event that this investigation should reveal only an inconsequential racemization, thus making the total proline content of gelatin and collagen about 17.5 per cent, one may easily calculate that the numerical ratios of proline to glycine to all amino acid residues are approximately 3:7:21. It is apparent that the proline residues cannot be distributed through the peptide chains of gelatin and collagen in a manner so that they recur regularly with a frequency of seven. It may become necessary, therefore, to assign a dual frequency to the distribution of proline in gelatin and collagen. However, we should like to reserve the discussion of the stoichiometry of gelatin and collagen pending the results of further investigations now in progress.

EXPERIMENTAL

General Remarks

When the solubility method is employed, the following precautions must be observed. The precipitating reagent used must be analytically pure. The recovered amino acid salt also should possess a high degree of purity, and its composition should be rigorously checked in every instance. The final concentration of the ions participating in the solubility product must be appreciable in comparison with the experimental errors involved in the determination of these concentrations. Suitable experimental conditions fulfilling these requirements must be found for each type of determination.

Finally, it should be emphasized that care must be taken to insure the establishment of a true equilibrium.

Sodium Dioxpyridate—This compound was prepared as described earlier (3). It was then dissolved in a minimum of water at room temperature, filtered, and salted-out by the addition of sodium acetate. After filtration it was suspended in about half its weight of ice-cold water, kept for 1 hour at 0°, and then sucked and dried on a porous plate to constant weight, first at 0° and then at room temperature. The salt was stored in the refrigerator.

Ammonium Rhodanilate—The salt was prepared as described previously (2). The product was then dissolved in cold methyl alcohol and, after addition of charcoal, filtered through a fluted filter. Ice water was added in several portions to the cooled filtrate. After filtration and washing with ice water, the recrystallization was repeated. The reagent was dried, protected from direct light on a porous plate, and stored in the refrigerator.

Determination of Glycine, l-Alanine, l-Leucine, and l-Proline As Dioxpyridates—As illustrative of the general case, the precipitation of *l*-proline with sodium dioxpyridate may be described in detail. 5.744 gm. of *l*-proline were dissolved in N HCl to a volume of 250 cc. To two 75 cc. portions of this solution were added 4.000 and 6.000 gm. of sodium dioxpyridate. The samples were first kept, in tightly stoppered flasks, at room temperature (22°) for 2 hours with shaking and then for 2 days at exactly 0° with frequent shaking. The precipitates were collected at 0° on weighed crucibles with sintered glass filter plates. The crucibles were dried in air at room temperature for 24 hours and then over $CaCl_2$ and NaOH to constant weight.

The data of this and other determinations will be found in Table I. There is also included an estimation of proline in presence of glycine and of leucine in presence of proline. In both determinations the purity of the precipitates was checked by amino nitrogen determinations. In the determination of proline the precipitate contained no amino nitrogen.

Determination of Proline As Rhodanilate—2.357 gm. of *l*-proline were dissolved in 75 cc. of N HCl and made up to a volume of 1 liter with water. The solution was cooled to 0°. Two cooled 205 cc. samples, each containing 483 mg. of proline, were then added to cooled solutions of 2.200 and 2.600 gm. samples of ammonium rhodanilate, each dissolved in 135 cc. of methanol. The mixtures were kept at exactly 0° for 16 hours, shaken at the same temperature for 4 hours, and again kept at 0° for 20 hours. The precipitates formed were collected at 0° on weighed crucibles with a sintered glass filter plate, each washed rapidly with 30 cc. of ice-cold water, and dried first in air and then over $CaCl_2$ to constant weight. The data from this experiment are found in Table I.

Preparation of Protein Hydrolysates—Protein hydrolysates prepared for constituent analysis by acid hydrolysis in the established manner cannot be employed for determination with the solubility method. The dark, colloidal decomposition products contained in such hydrolysates may inhibit the formation of precipitates in some cases, and in others the precipitates are highly contaminated. It is essential, therefore, to remove the decomposition products from the hydrolysate before attempting any analysis by the solubility method. It has been found that this may easily be accomplished by adding to the hydrolysate some pure $Cu(OH)_2$ and reprecipitating the copper with H_2S. The procedure has been successfully applied to hydrolysates of several proteins. The loss of amino acids, as measured by the determination of amino nitrogen, did not exceed 1.5 per cent and was, in most cases, negligible. The solutions resulting from this treatment have only a brownish yellow color and are optically clear.

Preparation of Collagen of Cattle Achilles Tendon—Cattle Achilles tendon was obtained in a fresh state from the slaughterhouse. The portion used was a strip extending 4 to 6 inches up the leg from the bifurcation at the hoof. Adhering fat and meat were removed, and the material was hashed by freezing with dry ice and passing through a grain grinder with large quantities of dry ice. In this manner the material remained frozen throughout the grinding process. The collagen was extracted at 0° for 2 weeks, with 10 per cent saline that was changed daily. The collagen was then successively extracted at 0° with M/15 Na_2HPO_4 for 3 days, with water until the extract gave a negative chloride test, and finally with ether for 2 weeks. The purified material was stored, still wet, at 0° with addition of toluene. A sample of this collagen was dried for 24 hours at 100° over P_2O_5.

1.182 gm. yielded 0.2191 gm. N, or 18.6%
1.351 " " 0.003 " ash as sulfate, or 0.22%

Determination of Glycine in Gelatin and Collagen—The determination of glycine with potassium trioxalatochromiate (7) does not lend itself to an evaluation from the point of view of the solubility product. The glycine compound precipitated contains glycine, potassium, and trioxalatochromiato ions in a rather complex ratio, rendering useless any mathematical treatment. It is possible, however, to apply to the glycine determination one of the checks worked out for the solubility method. By performing two comparative estimations, one with a certain amount of hydrolysate and the second with a smaller amount of hydrolysate to which is added a glycine solution of known content, one can detect in the determination any error exceeding 5 per cent. We feel justified, therefore, in accepting the results obtained with potassium trioxalatochromiate.

One precaution should be observed when potassium trioxalatochromiate is applied to the determination of glycine in protein hydrolysates. While the reagent has never been observed to precipitate any other natural amino acid except glycine, it does precipitate ammonia. The glycine content of the precipitate cannot, therefore, always be estimated solely by a determination of amino nitrogen. If considerable quantities of ammonia are present in the hydrolysate, part of it passes into the precipitate obtained with potassium trioxalatochromiate. The glycine content of this precipitate can be found by subtracting the ammonium nitrogen content of the precipitate from its total nitrogen content. The data from a series of determinations performed in this manner are found in Table II.

Determination of Proline in Gelatin and Collagen—50 cc. of a hydrolysate of pig skin gelatin (corresponding to 9.02 gm. of dry, ash-free protein) were mixed with 120 cc. of N HCl and the volume was made up with water to 1 liter. Three 275 cc. aliquots, each containing 2.48 gm. of protein, were added to solutions of 1.695, 2.001, and 2.301 gm. of ammonium rhodanilate, each dissolved in 175 cc. of methanol. The solutions were handled and the precipitates collected in the manner already described for the determination of pure proline, with the exception that these precipitates were not washed with water. The precipitates of *l*-proline rhodanilate weighed 1.283, 1.488, and 1.646 gm. The purity, determined by optical rotation, as described in the following paragraph, was 97.2, 96.9, and 96.7 per cent of the theory. From these data one may calculate, with the aid of Equation 2, the three values 442, 436, and 431 mg. for the *l*-proline content of the protein solutions used. The average, 436 mg., corresponds to 17.6 per cent of *l*-proline in pig skin gelatin. The results of all the determinations performed with varying amounts or concentrations of protein hydrolysate and with addition of proline are reported in Table III.

The purity of proline rhodanilate precipitates obtained in these determinations cannot be estimated accurately by elementary analysis alone. A more sensitive method has therefore been developed. Use was made of the fact that *l*-proline has a distinctively high negative rotation. The procedure was as follows:

A 300 mg. sample of a proline rhodanilate, the purity of which was to be ascertained, was shaken for ½ hour at room temperature with 1 cc. of glacial acetic acid and 0.15 cc. of dimethylaniline in a tared, stoppered flask. Then 2 cc. of water were added, the flask and contents were weighed and shaken vigorously, and the precipitated dimethylaniline rhodanilate was removed by filtration. The filtration was carried out under a difference in pressure of 6 mm. of Hg, with the use of the apparatus portrayed in Fig. 2. To the filtrate, which at this point was still colored, a few mg. of charcoal were added. The solution was then filtered again in the same way through a dry filter. The optical rotation of the filtrate was determined in a 1 dm. tube.

As a reference standard, pure proline rhodanilate was subjected to this procedure. 300 mg. of substance in 3.19 gm. of solvents gave α (1 dm.) = $-1.52°$ (average of eight determinations).

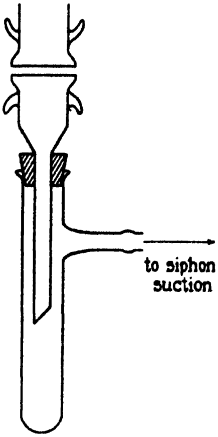


Fig. 2. Filter used in the decomposition of proline rhodanilate. A disk of hardened filter paper is inserted between the two halves of the filter, which are then clamped together by springs attached to the glass arms on either side.

In the calculation of the proline content of a proline rhodanilate sample obtained from the analytical procedures described, the following formula was used.

$$p = \frac{\alpha \times S \times 300 \times 57}{-1.52° \times 3.19 \times R}$$

where p = proline present (in mg.)
α = observed rotation (in degrees)
S = total weight of solvents (acetic acid, dimethylaniline, water, in gm.)
R = weight of sample of proline rhodanilate in mg.

300 mg. of pure proline rhodanilate correspond to 57.0 mg. of proline. The proline content of rhodanilate precipitates obtained in the course of analyses by the solubility method should closely correspond to this value. Determinations of the proline content of mixtures of proline rhodanilate and ammonium rhodanilate of known proportions indicated that the above procedure yields results accurate to ±1.5 per cent.

BIBLIOGRAPHY

1. Stein, W. H., Niemann, C., and Bergmann, M., *J. Am. Chem. Soc.*, **60**, 1703 (1938).
2. Bergmann, M., *J. Biol. Chem.*, **110**, 471 (1935).
3. Bergmann, M., *J Biol. Chem.*, **122**, 569 (1938).
4. Astbury, W. T., *Nature*, **140**, 968 (1937).
5. Abderhalden, E., and Voitinovici, A., *Z. physiol. Chem.*, **52**, 348 (1907).
6. Fischer, E., *Ber. chem. Ges.*, **39**, 593 (1906).
7. Bergmann, M., and Niemann, C., *J. Biol. Chem.*, **122**, 577 (1938).
8. Dakin, H. D., *J. Biol. Chem.*, **44**, 499 (1920).
9. Astbury, W. T., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **2**, 15 (1934).

SULFHYDRYL GROUPS IN PROTEINS

II. EDESTIN, EXCELSIN, AND GLOBIN IN SOLUTIONS OF GUANIDINE HYDROCHLORIDE, UREA, AND THEIR DERIVATIVES

By JESSE P. GREENSTEIN

(*From the Department of Physical Chemistry, Harvard Medical School, Boston*)

(Received for publication, January 19, 1939)

Egg albumin in the native, unaltered state, does not give tests characteristic of sulfhydryl groups. When, however, this protein is treated in any one of several ways, such as by heat (8, 9, 17), ultraviolet irradiation (10), shaking (10), or by solution in urea or other amides (7), free sulfhydryl groups make their appearance. The amount of free —SH groups appearing in egg albumin through the action of urea, guanidine hydrochloride, and their derivatives has been estimated (6). Considerable differences in the number of such groups liberated, which depend on the nature and the concentration of the reagent used, were noted.

The investigation of these groups has been extended to a number of proteins whose molecular weights have been reported to be smaller in urea solutions than in water. The three seed globulins, edestin, excelsin, and amandin, have practically the same molecular weight in water (4, 5, 15, 16). Their molecular weights in 4 M urea are also almost the same (4), but are reported to be only one-eighth as great as in water. Burk and Greenberg (5) showed that the molecular weight of horse hemoglobin in urea solution was 34,000, a value just half the molecular weight of this protein in water. The results of these investigators were confirmed by Wu and Yang (18) and by Steinhardt (14), the latter showing further that the molecular weight in acetamide solutions was comparable to that in urea.

It has been suggested that the difference in the molecular weights of certain proteins in water and in urea is due in part to a scission of the S—S linkages of the protein in the latter solvent with the subsequent appearance of —SH groups (4).[1] It seemed desirable, therefore, to study these groups in proteins dissolved in this and related solvents.

EXPERIMENTAL

Preparation of Proteins—Edestin was prepared from ground hemp seed by the method of Svedberg and Sjögren (16). Five different preparations were employed. The protein was dissolved in several concentrations in 10 per cent sodium chloride solution. The concentrations of protein were estimated by nitrogen analyses, assuming Bailey's value of 18.4 for edestin nitrogen (3). Excelsin was prepared from Brazil nuts by a slight modification of the method of Burk (4). Three different preparations were used. Like edestin, the protein was dissolved in varying concentrations in 10 per cent sodium chloride. The concentrations of protein were estimated, assuming Osborne's value of 18.3 per cent nitrogen (12). Amandin was prepared from ground almonds according to the method of Burk (4) and dissolved in 10 per cent sodium chloride. Osborne's value of 19.3 per cent nitrogen for this protein was used to estimate the concentration (12).

Globin was prepared from denatured horse globin by the method of Anson and Mirsky (1).[2] The solution of globin, during the dialysis from ammonium sulfate, deposited a copious precipitate of denatured material. The filtered solution, containing native globin, amounted to 3.70 per cent in concentration from both nitrogen analyses (16.9 per cent for globin N) and dry weight determinations. Studies were made on fresh solutions, because the denaturation of this protein, under most conditions, is fairly rapid.

Estimation of —SH Groups— A convenient method for the estimation of protein —SH groups consists in titrating the protein with porphyrindin, a method first introduced by Kuhn and Desnuelle (8). This dye is a powerful oxidizing agent and reacts rapidly and stoichiometrically with sulfhydryl groups at a neutral reaction and at room temperature. When the dye is added to an aqueous solution of egg albumin (6), edestin, excelsin, amandin, or globin, no apparent reduction occurs. When, however, these proteins, with the exception of amandin, are dissolved in solutions of urea, guanidine hydrochloride, and *some* of their derivatives, free —SH groups appear, and a certain amount of the dye added to such solutions becomes reduced to the colorless leuco form (6).[3] For convenience, the amount of dye reduced by the protein is expressed in terms of its equivalent of cysteine per 100 gm. of protein (6, 8).

---

[1] It has been shown that none of the reagents such as urea and guanidine hydrochloride, which act on certain proteins in such a way that —SH groups appear, has any effect on the following complex peptides of cystine: cystinylcystine, bisanhydrocystinylcystine, cystinyldidiglycine, and cystinyldiglycine (6).

[2] Wu and Yang (18) have shown that the globins of various species have the same properties in urea as the parent hemoglobins.

The titrations were conducted by adding to the solution of protein in urea, guanidine hydrochloride, and to their derivatives, a solution of the porphyrindin dye until a negative nitroprusside reaction was obtained. All determinations were performed in sextuplicate. The temperature was 30°. Before and after every series of measurements, the dye was titrated against a standard cysteine solution (6). For the studies with the seed globulins in 10 per cent sodium chloride solution the dye was used at a lower concentration than in the studies with globin, which were conducted in water. The lower concentration of dye in the former case was employed partly because of its lower solubility in salt solution, and partly because of the relatively small number of sulfhydryl groups which appeared in these proteins.

The first experiments were concerned with studying the effect of urea and guanidine hydrochloride in equimolar amounts on different concentrations of the proteins. No effect whatever of these reagents was found with amandin. Neither the preparation of amandin made in this laboratory, nor one which had been made by T. B. Osborne, gave a sulfhydryl reaction in either urea or guanidine hydrochloride.[4] The fact that no reduction of the porphyrindin dye occurred with this protein in solutions of urea and guanidine hydrochloride indicated that the dye was unaffected by any groups other than sulfhydryl (11), which may have been liberated by the reagents.[5] The results on edestin, excelsin, amandin, and globin are reported in Table I, expressed as cysteine per 100 gm. of protein.

---

[3] The presence of free sulfydryl groups in the protein can be followed by a positive nitroprusside reaction. After the addition of a sufficient amount of dye to the protein, this reaction becomes negative.

[4] I am greatly indebted to Dr. Vickery for an Osborne preparation of amandin and to Dr. Hitchcock for a sample of edestin.

Edestin, excelsin, and globin were further investigated in solutions of varying concentration of guanidine hydrochloride. The very strong effect of this reagent is revealed in Table II, the results being comparable to those obtained on egg albumin (6).

TABLE I

Effect of Varying Concentrations of Proteins

In each determination 2.0 cc. of protein solution were used. The urea added was 2.0 gm.; the guanidine hydrochloride added was 3.2 gm. In edestin, excelsin, and amandin solutions the dye was used in 1.16×10^{-3} M concentration (0.0325 per cent) in 10 per cent NaCl; in the globin solutions the dye was used in 2.32×10^{-3} M concentration (0.065 per cent) in water.

Protein	Per cent protein in concentration	Dye		Cysteine	
		Urea	Guanidine HCl	Urea	Guanidine HCl
		mM × 10⁴	*mM × 10⁴*	*per cent*	*per cent*
Edestin	0.98	2.66	4.17	0.32	0.50
	1.47	4.17	6.42	0.34	0.51
	1.69	4.75	7.29	0.35	0.50
	1.96	5.33	8.34	0.32	0.50
	2.45	6.95	10.42	0.34	0.51
Excelsin	5.30	3.59	7.54	0.08	0.17
	8.20	5.09	12.74	0.07	0.17
	10.90	7.07	14.48	0.08	0.16
	11.00	6.40	15.18	0.07	0.16
Amandin	10.50	<0.07	<0.07	<0.01	<0.01
Globin	1.85	2.71	8.13	0.19	0.56
	2.77	4.06	12.18	0.19	0.56
	3.70	5.42	16.26	0.19	0.56

In Table III is described the effect of various derivatives of urea and guanidine hydrochloride on the proteins. For comparison, the data on egg albumin (6) are included. All the reagents were used in equimolar concentrations.

---

[5] In the dried, solid state, the dye appears to be quite stable. Material which had stood for several months at room temperature showed no loss in titer in fresh solution.

TABLE II

Effect of Varying Concentrations of Guanidine Hydrochloride on Edestin, Excelsin, and Globin

Edestin was employed in 1.96 per cent solution, excelsin in 8.20 per cent, and globin in 3.70 per cent. In each test 2.0 cc. of protein solution were used. In edestin and excelsin solutions the dye was used in 1.16×10^{-3} M concentration (0.0325 per cent) in 10 per cent NaCl; in the globin solutions the dye was used in 2.32×10^{-3} M concentration (0.065 per cent) in water.

Guanidine HCl added	Edestin		Excelsin		Globin	
	Dye	Cysteine	Dye	Cysteine	Dye	Cysteine
gm.	*mM × 10⁴*	*per cent*	*mM × 10⁴*	*per cent*	*mM × 10⁴*	*per cent*
0.2	0.0	0	0.0	0	0.0	0
0.4	2.32	0.14	3.48	0.05	4.18	0.14
0.8	8.11	0.50	12.76	0.18	16.72	0.56
1.2	8.11	0.50	12.76	0.18	16.72	0.56
1.6	8.11	0.50	12.76	0.18	16.72	0.56
2.0	8.11	0.50	12.76	0.18	16.72	0.56
2.4	8.11	0.50	12.76	0.18	16.72	0.56
2.8	8.11	0.50	12.76	0.18	16.72	0.56
3.2	8.11	0.50	12.76	0.18	16.72	0.56

TABLE III

Effect of Urea, Guanidine Hydrochloride, and Their Derivatives on Proteins

The concentration of the protein solutions was as follows: egg albumin 5.45 per cent, edestin 2.45 per cent, excelsin 8.2 per cent, and globin 3.70 per cent.

Reagent	Amount of reagent added per cc. protein solution	Cysteine			
		Egg albumin	Edestin	Excelsin	Globin
	gm.	*per cent*	*per cent*	*per cent*	*per cent*
Urea	1.00	1.00	0.34	0.07	0.19
N-Methylurea	1.23	1.02	<0.03	<0.01	<0.03
O-Methylisourea hydrochloride	1.84	1.05	0.17	<0.01	<0.03
Guanidine hydrochloride	1.60	1.20	0.51	0.18	0.56
Methylguanidine hydrochloride	1.82	1.19	0.16	0.07	0.56
as-Dimethylguanidine hydrochloride	2.00	0.75	<0.03	<0.01	<0.03
Acetamide	1.0	<0.05	<0.03	<0.01	<0.03

DISCUSSION

As in egg albumin, the proportion of sulfhydryl groups liberated in edestin, excelsin, and globin is apparently independent of the protein concentration (Table I). The behavior of edestin, excelsin, and globin toward varying concentrations of guanidine hydrochloride (Table II) is also strikingly similar to that of egg albumin (6) in that guanidine hydrochloride reaches its maximum effect at a comparatively low concentration.

The comparison of the effects of various derivatives of urea and of guanidine hydrochloride in Table III reveals not only the uniformly stronger action of guanidine hydrochloride over urea for all of the proteins studied, but also the relatively weak action of the methylated ureas on edestin, excelsin, and globin. In egg albumin, on the other hand, the methylated ureas are as effective as the parent substance. Methylguanidine hydrochloride exerts a weak effect on edestin and excelsin, but in globin, as in egg albumin, its action is equal to that of guanidine hydrochloride itself. Dimethylguanidine hydrochloride and acetamide have little or no effect on edestin, excelsin, or globin.

Osborne's early investigations on the analytical data for edestin and excelsin led to a value for the molecular weight of the former of 14,523 and for the latter of 14,738, or some multiple of these (12). Osborne's value (12) as well as that of Zahnd and Clarke (19) for the alkali-labile sulfur in edestin is 0.35 per cent. The alkali-labile sulfur in excelsin is also 0.35 per cent (12). These results lead to the assumption of 3 atoms of alkali-labile sulfur in each protein of molecular weight between 28,000 and 29,000.[6]

When edestin is dissolved in solutions of guanidine hydrochloride, 0.51 per cent cysteine appears (Table III). On the basis of a molecular weight of 28,000, this amounts to 1 mole of cysteine per mole of protein. In excelsin dissolved in guanidine hydrochloride, the cysteine which appears amounts to 0.17 per cent. On the basis of a molecular weight of 28,000, this amounts to 0.3 mole of cysteine per mole of protein, or better, 1 mole of cysteine per mole of protein of molecular weight 84,000. Under similar conditions, therefore, the cysteine which appears in guanidine hydrochloride solutions of edestin amounts to 3 times that which appears in excelsin.

---

[6] Amandin, according to Osborne, has 0.22 per cent of alkali-labile sulfur (12). On the basis of a molecular weight of 28,000 to 29,000 (12), there would appear to be 2 atoms of alkali-labile sulfur per mole of protein. It is probable that all the alkali-labile sulfur of this protein belongs to cystine.

Schulz reported for horse globin a value of 0.20 per cent for the alkali-labile sulfur (13). On the basis of a molecular weight for globin of approximately 66,000, there would appear to be 4 atoms of alkali-labile sulfur per mole of protein. When this protein is dissolved in guanidine hydrochloride solutions, there appears 0.56 per cent cysteine, or 3 moles of cysteine per mole of protein.[7]

To summarize the results obtained thus far on the proteins in guanidine hydrochloride solutions: *in equal concentrations of this reagent, the cysteine determined amounts in egg albumin to two-thirds, in edestin to one-third, in excelsin to one-ninth, and in globin to three-fourths of the alkali-labile sulfur.*

Although the molecular weights in urea of edestin, excelsin, and amandin appear to be very nearly the same (4), the proportion of cysteine which appears in the three proteins is very different (Table I). Hemoglobin has the same molecular weight in either urea or in acetamide, namely half that found in water (14); in globin, however, cysteine appears in urea, not in acetamide. It seems, therefore, that no obvious relation exists between the differences of molecular weight in different solvents and the appearance of sulfhydryl groups. The latter in all probability exist as part of the cysteine residues within the native protein, and do not owe their origin to scission of disulfide linkages.

It is evident that the present results may have broad implications for the problem of protein denaturation. The number of sulfhydryl groups liberated, which may be taken as a measure of the change in protein configuration, is clearly different according to the nature and the concentration of the individual denaturing agent. Further studies of the effect of urea, guanidine hydrochloride, and their derivatives on other properties of the proteins are at present under way.

SUMMARY

1. Edestin, excelsin, amandin, and globin in aqueous solution do not give tests characteristic of sulfhydryl groups. When these

---

[7] Anson and Mirsky (2) found the cysteine content of acid-acetone globin to be 0.38 per cent. This amount of cysteine is equivalent to 2 moles per mole of protein.

proteins, with the exception of amandin, are treated with urea, guanidine hydrochloride, and certain of their derivatives, free sulfhydryl groups appear. The estimation of these groups is accomplished by the use of the porphyrindin dye. The results are expressed in terms of cysteine.

2. The proportion of cysteine which appears is independent of the protein concentration. Guanidine hydrochloride exerts the strongest action of all the reagents studied, and reaches its maximum effect at a comparatively low concentration. The methylated ureas as well as methylguanidine hydrochloride have a weak effect on edestin and excelsin. In globin, methylguanidine hydrochloride is as effective as the parent substance. Dimethylguanidine hydrochloride and acetamide have little or no effect on edestin, excelsin, and globin.

3. The relation of the cysteine which appears in solutions of guanidine hydrochloride to the alkali-labile sulfur of the proteins has been considered.

4. There does not appear to be any correlation between the differences in molecular weight of these proteins in water and in solutions of certain amides and the amount of cysteine which may appear in the latter solvents.

BIBLIOGRAPHY

1. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **13**, 471 (1929-30).
2. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **19**, 439 (1935-36).
3. Bailey, K., *Biochem. J.*, **31**, 1396 (1937).
4. Burk, N. F., *J. Biol. Chem.*, **120**, 63 (1937).
5. Burk, N. F., and Greenberg, D. M., *J. Biol. Chem.*, **87**, 197 (1930).
6. Greenstein, J. P., *J. Biol. Chem.*, **125**, 501 (1938).
7. Hopkins, F. G., *Nature*, **126**, 328, 383 (1930).
8. Kuhn, R., and Desnuelle, P., *Z. physiol. Chem.*, **251**, 14 (1938).
9. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **18**, 307 (1935-36).
10. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **19**, 427 (1935-36).
11. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **19**, 451 (1935-36).
12. Osborne, T. B., *J. Am. Chem. Soc.*, **24**, 39, 140 (1902).
13. Schulz, F. N., *Z. physiol. Chem.*, **25**, 16 (1898).
14. Steinhardt, J., *J. Biol. Chem.*, **123**, 543 (1938).
15. Svedberg, T., *Chem. Rev.*, **20**, 81 (1937).
16. Svedberg, T., and Sjögren, B., *J. Am. Chm. Soc.*, **52**, 279 (1930).
17. Todrick, A., and Walker, E., *Biochem. J.*, **31**, 292 (1937).
18. Wu, H., and Yang, E.-F., *Chinese J. Physiol.*, **6**, 51 (1932).
19. Zahnd, H., and Clarke, H. T., *J. Biol. Chem.*, **102**, 171 (1933).

STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

XI. THE SYNTHESIS OF DIGLYCYL-*l*-CYSTINE

By JESSE P. GREENSTEIN

(*From the Department of Physical Chemistry, Harvard Medical School, Boston*)

(Received for publication, January 19, 1939)

In a previous communication, the use of cystinyldidiglycine and cystinyldiglycine as substrates respectively for aminopolypeptidase and dipeptidase was described (3). An important advantage in the use of these molecules lay in the fact that *l*-cystine crystallized out of the enzyme mixture during the hydrolysis, while the peptide itself remained in solution. In both of these peptides, the cystine component carried the free amino groups. It was thought desirable to study the effect of the peptidases on a soluble cystine peptide in which the cystine component carried the free carboxyl groups. Diglycyl-*l*-cystine was therefore synthesized and obtained in the form of long, prismatic crystals.[1] This substance was incubated with an extract of crude erepsin and with carboxypeptidase. The former enzyme rapidly hydrolyzed the peptide; the latter had no apparent effect.

EXPERIMENTAL

Dicarbobenzoxyglycyl-l-Cystine—25.2 gm. of carbobenzoxyglycine (1) were converted to the acid chloride by shaking in dry ethyl ether with 26.8 gm. of pulverized phosphorus pentachloride. The ether solution was rapidly condensed *in vacuo* and the residual syrup washed three times with chilled petroleum ether. The syrup was then taken up in a little dry ethyl ether and added in four portions with shaking to a cooled solution of 25 gm. of *l*-cystine in 300 cc. of 1 N KOH. The mixture was extracted with ether and acidified to a Congo blue reaction with 5 N HCl. A syrup appeared which was immediately taken up in ethyl acetate. The ethyl acetate solution was twice washed with very dilute HCl and then once with water, dried for a few minutes over anhydrous sodium sulfate, and evaporated *in vacuo* to about a third of the original volume. On chilling overnight, a copious crystallization of the coupling product occurred in the form of needles. M. p. 142°. The yield amounted to 15 gm.

---

[1] Fischer and Suzuki (2) described an amorphous preparation of diglycylcystine which had no constant melting point.

$C_{26}H_{30}O_{10}N_4S_2$ (622). Calculated, N 9.0, S 10.3; found, N 8.9, S 10.1

Diglycyl-l-Cystine—25 gm. of the coupling product were dissolved in 250 cc. of liquid ammonia (4). The solution was treated with small pieces of metallic sodium with stirring until a blue color appeared. After the ammonia had been allowed to evaporate off spontaneously, the container was evacuated at the water pump for several hours. The residue was taken up in cold water and treated with dilute sulfuric acid until the reaction was slightly acid to litmus. Mercuric sulfate reagent was added to precipitate the peptide. The mercury salt was washed with water several times at the centrifuge, decomposed with hydrogen sulfide, and the precipitation procedure with mercuric sulfate repeated. The final solution was treated with a concentrated baryta solution until slightly alkaline, and the barium sulfate removed at the centrifuge. A few crystals of iron oxide were added to the solution and air was bubbled through until a negative nitroprusside reaction was attained. The solution was then treated with norit, quantitatively freed of barium by the addition of dilute sulfuric acid, and evaporated *in vacuo* almost to dryness. Addition of alcohol to the concentrate caused the precipitation of the oxidized peptide as a gelatinous mass. The latter was taken up several times in cold water and precipitated each time as a gelatinous mass with ethanol. Crystallization of the peptide was finally accomplished by heating the freshly precipitated mass, which still enclosed a good deal of alcohol, with a very small amount of water. Soon after the solid dissolved, the peptide began to crystallize from the hot solution in the form of long prisms. The mixture was chilled for several hours, and the peptide filtered off and washed with alcohol and ether. Yield 8 gm. M. p. 232° with decomposition.

The peptide was quite soluble in water. It crystallized with 1 molecule of water. 50 mg. heated at 78° and 1 mm. for 6 hours lost 2.4 mg. in weight; calculated for 1 molecule of water, 4.8 per cent; found 4.8 per cent.

$[\alpha]_D^{24} = -108°$ for a 0.75 per cent solution of the peptide in 1 N HCl. 150 mg. of the peptide in 10 cc. of 2 N HCl were boiled under the reflux for 2 hours. 65 mg. of *l*-cystine were recovered from the hydrolysis mixture. The amino acid possessed an $[\alpha]_D^{24} = -215°$ for a 0.65 per cent solution in 1 N HCl. Little if any racemization had apparently occurred during the synthesis of the peptide.

$C_{10}H_{18}O_6N_4S_2 + 1\ H_2O$ (372).

Calculated.	C 32.2,	H 5.3,	N 15.0,	S 17.2
Found.	" 32.0,	" 5.3,	" 14.6,	" 17.0

Effect of Peptidases—50 mg. of the peptide in 10 cc. of solution, incubated at pH 7.0 and at 30° with a glycerol extract of swine intestinal mucosa, were hydrolyzed after 18 hours to 92 per cent. After 1 hour the *l*-cystine began to separate in the form of hexagonal plates. The cystine was finally filtered off and estimated as before (3). $[\alpha]_D^{22}$ for the amino acid was −212°. A similar solution of the peptide, incubated with a carboxypeptidase preparation (3), showed no hydrolysis whatever under conditions whereby an equivalent amount of chloroacetyl-*l*-tyrosine was hydrolyzed to 83 per cent.

SUMMARY

The synthesis of crystalline diglycyl-*l*-cystine has been described. The peptide is rapidly hydrolyzed by intestinal erepsin, with the simultaneous crystallization from the mixture of hexagonal plates of *l*-cystine. Carboxypeptidase has no apparent effect on the molecule.

BIBLIOGRAPHY

1. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1192 (1932).
2. Fischer, E., and Suzuki, U., *Ber. chem. Ges.*, **37**, 4575 (1904).
3. Greenstein, J. P., *J. Biol. Chem.*, **124**, 255 (1938).
4. Loring, H. S., and du Vigneaud, V., *J. Biol. Chem.*, **111**, 385 (1935).

ASPARTYLHISTIDINE

By JESSE P. GREENSTEIN and FRIEDRICH W. KLEMPERER

(*From the Departments of Physical Chemistry and of Biological Chemistry, Harvard Medical School, Boston*)

(Received for publication, January 19, 1939)

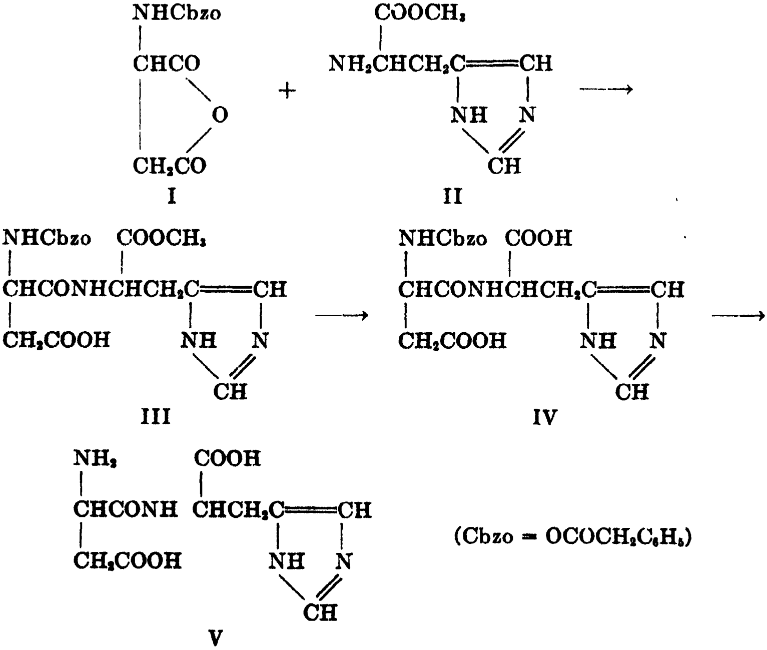
The peptides are of particular interest because of their intermediate position between the amino acids and the proteins. The study of the properties of the more complex peptides may be regarded as revealing certain of the properties of the proteins. In order to study peptides containing several free dissociating groups, a number of tetravalent amino acids and peptides have been synthesized (2, 4, 5-7).

Amino acids and peptides with two positively and two negatively charged groups may be classified according to the spatial relations of these groups. If we assign + to represent a positively charged group, and − a negatively charged group, lysylglutamic acid is expressed as + + − − (2, 4, 8). Cystinyldiglycine (5, 7, 12) and cystinyldidiglycine (5, 7) are represented by − + + −. On the other hand, ϵ,ϵ'-diaminodi-(α-thio-*n*-caproic acid) (5, 7), ϵ,ϵ'-diguanidodi-(α-thio-*n*-caproic acid) (5, 7), and diglycylcystine (6) are expressed as + − − +. One type of tetrapolar configuration has not been investigated; namely, that in which the signs of the electrically charged groups alternate. Accordingly, α-aspartylhistidine has been synthesized and is described in the present communication. The configuration of this molecule according to the above scheme is − + − +.

Recently du Vigneaud and Hunt (14) have described the preparation of β-aspartylhistidine. Inasmuch as in this molecule the amino and carboxyl groups of the aspartyl radical are on the same carbon atom, its configuration would be represented by ± − +. Through the generous cooperation of Dr. du Vigneaud, a sample of the β-aspartylhistidine has been made available to us. The apparent dissociation constants of the α- and the β-peptides have been measured and compared. The differences in the constants of the two substances were found to be consistent with expectation on the basis of their structures.[1]

EXPERIMENTAL

The α-aspartylhistidine was prepared from N-carbobenzoxy-*l*-aspartic acid anhydride (I) which was allowed to condense with an excess of *d*-histidine methyl ester (II) in dry chloroform. Inasmuch as the condensation product is a dipolar ion, it precipitates out immediately from chloroform solution. The product, N-carbobenzoxy-*l*-aspartyl-*d*-histidine methyl ester (III) was then saponified and the solution was subsequently acidified, whereby the crystalline free acid (IV) was formed. On catalytic hydrogenation in the presence of palladium, the peptide *l*-aspartyl-*d*-histidine (V) was obtained. The reactions may be illustrated in the accompanying formulas.



(Cbzo = $OCOCH_2C_6H_5$)

---

[1] The β-peptide of du Vigneaud and Hunt is *l*-aspartyl-*l*-histidine (14). The α-peptide described in the present paper is *l*-aspartyl-*d*-histidine. The apparent dissociation constants as well as many other physicochemical properties of such substances are independent of their optical properties.

N-Carbobenzoxy-l-Aspartyl-d-Histidine Methyl Ester (*III*)—10.3 gm. of *d*-histidine methyl ester dihydrochloride were dissolved in 125 cc. of hot dry methanol and chilled. To this solution were quickly added 29 cc. of 2.74 N sodium methylate and subsequently 100 cc. of dry ether. After having stood for a few minutes, the mixture was filtered through infusorial earth and the clear filtrate was evaporated to a syrup *in vacuo* at room temperature. Traces of alcohol were removed by three distillations with dry chloroform. The syrup was taken up in 50 cc. of chloroform and filtered through norit from a little NaCl. To the chilled filtrate were added 3 gm. of N-carbobenzoxy-*l*-aspartic acid anhydride (1) in small portions and with shaking. Almost immediately after the last portion of the anhydride had been dissolved, the coupling product separated in a gelatinous mass. The mixture was shaken by machine for another half hour and was then allowed to stand at room temperature overnight. The product was filtered, washed with chloroform, and shaken by machine with ethyl acetate. This treatment rendered the material granular. Dried in air, the product weighed 4.1 gm. The melting point was 95–105° with decomposition.

$C_{19}H_{22}O_7N_4$ (418.2). Calculated, N 13.4; found, N 13.2

N-Carbobenzoxy-l-Aspartyl-d-Histidine (*IV*)—4 gm. of the coupling product were dissolved with shaking in 25 cc. of 1 N KOH and the solution was allowed to stand for 45 minutes at room temperature. It was then filtered and treated with 2 cc. of glacial acetic acid. On evaporation and scratching, the solution set to a mass of long needles. The crystals were filtered, washed with a little ice water, and dried. Yield, 1.7 gm.; m.p. 171°, with decomposition. The substance was already sufficiently pure for the next step.

$C_{18}H_{20}O_7N_4$ (404.2). Calculated, N 13.9; found, N 13.8

l-Aspartyl-d-Histidine (*V*)—1.6 gm. of the substance previously described were ground fine and suspended in 50 cc. of water which contained a few drops of glacial acetic acid. The mixture was then hydrogenated catalytically in the presence of 0.5 gm. of palladium. After half an hour all of the material had gone into solution, but the shaking was continued for another hour. The peptide was recovered from the solution after evaporation *in vacuo* and was recrystallized in the form of long prisms from hot water-ethanol. The yield was 1.1 gm. or nearly quantitative. M.p.

210°. The substance gave a positive Pauly diazo reaction. Its optical rotation was rather small, $[\alpha]_D^{30} = -6.0°$ for a 1 per cent solution in water. On heating at 78° for 4 hours at 4 mm. pressure, the peptide lost 11.5 per cent in weight; calculated for 2 molecules of crystal water, 11.7 per cent.

$C_{10}H_{14}O_5N_4 + 2H_2O$ (306).	Calculated.	C 39.2, H 5.9, N 18.3
	Found.	" 39.0, " 6.0, " 18.0

Electrometric Titration of α- and β-Aspartylhistidine—The isomeric peptides were titrated at 38° in 0.1 M KCl with the use of the hydrogen electrode. pH determinations were referred to standard acetate which has a pH value of 4.65 according to Hitchcock and Taylor (10). The calculation of the apparent dissociation constants followed in general the procedure of Hastings and Van Slyke (9). Corrections for hydrogen and hydroxyl ion activities were made, with activity coefficients of 0.84 and 0.81 respectively (11). A pK_w value of 13.55 was determined under the conditions of our procedure.

The constants of these compounds, as well as those of the isomeric α- and β-asparagines reported by Melville and Richardson (13) are included in Table I. In the last column the isoelectric points for each compound are given. The titration curves of the peptides at 38° are represented in Fig. 1; the points are the experimentally determined values; the curves are calculated from the constants given in Table I.

The apparent dissociation constants may be allocated to the various groups of the peptides as follows: pK'_1 and pK'_2 represent the two carboxyl groups, pK'_3 the imidazole ring, and pK'_4 the amino group. Inasmuch as pK'_2 has approximately the same value in the α- and the β-aspartylhistidine, it presumably represents the dissociation of the carboxyl group of the histidine portion of the peptide. On the other hand, since the carboxyl group on the aspartyl portion of the peptides is in a different position when the peptide is in the α- or in the β-configuration, its dissociation constant might be expected to vary. Since pK'_1 is distinctly different in the two peptides, it may be assumed to represent the dissociation of the free aspartyl carboxyl group. This difference would be reflected also in the values for the amino group, since the latter also belongs to the aspartyl portion of the peptides, and this is indeed the case (Table I).

The amino and carboxyl groups of the aspartyl portion of the β-peptide are on the same carbon atom. In the α-peptide, these same groups are located on contiguous carbon atoms. Exactly the same configurations exist, respectively, in β-asparagine and α-isoasparagine. The constants found by Melville and Richardson (13) for these substances show differences entirely consistent with those of the aspartyl peptides. The pK$'_1$ values for α-aspartylhistidine and for α-isoasparagine are both more alkaline; the pK$'_4$ values, both more acid than the corresponding values repectively for β-aspartylhistidine and for β-asparagine (Table I).

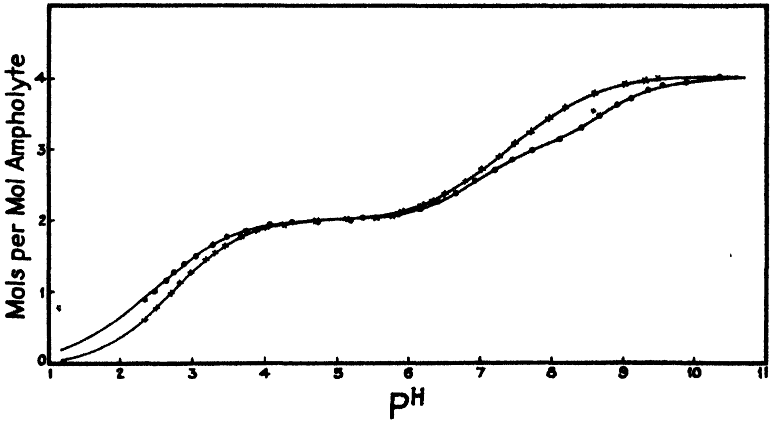


Fig. 1. The titration curves at 38° of the isomeric aspartylhistidines. The points are those experimentally determined; the curves are based on the constants given in Table I. The crosses refer to the α-peptide, the dots to the β-peptide.

TABLE I

Dissociation Constants for α- and β-Aspartyl Peptides

Peptide	Temperature	pK$'_1$	pK$'_2$	pK$'_3$	pK$'_4$	Isoelectric point
	°C.					
α-Isoasparagine	25	2.97			8.02	5.49
β-Asparagine	25	2.02			8.80	5.41
α-Aspartylhistidine	38	2.45	3.02	6.82	7.98	4.92
β-Aspartylhistidine	38	1.93	2.95	6.93	8.72	4.94

The values of pK'_3 for the imidazole groups in these peptides are more alkaline than have been observed previously in molecules of this kind (3, 4). This indicates, moreover, that it is possible for histidine within the protein molecule to combine with acid well above a pH of 7, which must be taken into account in the consideration of the titration curves of proteins.

SUMMARY

1. The neutral tetravalent peptide α-aspartylhistidine has been synthesized. In this molecule there is an alternation in the positions of the positively and negatively charged groups.

2. The apparent dissociation constants of the active groups of this molecule as well as those of the isomeric β-aspartylhistidine have been measured and compared. The significance of certain of the results for the problem of the interpretation of protein titration curves is considered.

BIBLIOGRAPHY

1. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65,** 1192 (1932).
2. Bergmann, M., Zervas, L., and Greenstein, J. P., *Ber. chem. Ges.*, **65,** 1692 (1932).
3. Cohn, E J., *Ergebn. Physiol.*, **33,** 781 (1931).
4. Greenstein, J. P., *J. Biol. Chem.*, **101,** 603 (1933).
5. Greenstein, J. P., *J. Biol. Chem.*, **109,** 529 (1935); **124,** 255 (1938).
6. Greenstein, J. P., *J. Biol. Chem.*, **128,** 241 (1939).
7. Greenstein, J. P., Klemperer, F. W., and Wyman, J., *J. Biol. Chem.*, **125,** 515 (1938).
8. Greenstein, J. P., Wyman, J., and Cohn, E. J., *J. Am. Chem. Soc.*, **57,** 637 (1935).
9. Hastings, A. B., and Van Slyke, D. D., *J. Biol. Chem.*, **53,** 269 (1922).
10. Hitchcock, D. I., and Taylor, A. C., *J. Am. Chem. Soc.*, **60,** 2710 (1938).
11. Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, New York and London, 382 (1923).
12. Loring, H. S., and du Vigneaud, V., *J. Biol. Chem.*, **111,** 385 (1935).
13. Melville, J., and Richardson, G. M., *Biochem. J.*, **29,** 187 (1935).
14. du Vigneaud, V., and Hunt, M., *J. Biol. Chem.*, **125,** 269 (1938).

THE PROMOTING EFFECT OF ALUMINUM, CHROMIUM, AND THE RARE EARTHS IN THE SUCCINIC DEHYDROGENASE-CYTOCHROME SYSTEM

By B. L. HORECKER, ELMER STOTZ, and T. R. HOGNESS

(From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago)

(Received for publication, November 28, 1938)

In the course of an investigation on the succinic dehydrogenase-cytochrome enzyme system, γ-aluminum hydroxide was used in an attempt to isolate certain of its components. The inconsistency of the results and the increased total activity obtained by this procedure suggested that some important factor was introduced by this treatment. Studies with such a factor in view showed clearly that aluminum ion addition in very small concentrations accelerated the aerobic oxidation of succinate markedly. Subsequent studies showed that aluminum is not unique in this accelerating property, but that it may be substituted by trivalent chromium and certain rare earths but not by ferric ion or by divalent ions. However, spectroscopic examination showed that of these metals only aluminum was present in the enzyme preparations in amounts sufficient to be of any significance. The following is a report of these findings.

EXPERIMENTAL

After it was determined qualitatively that the addition of aluminum ion, even in as small a concentration as that afforded by a saturated aluminum hydroxide solution, accelerated the rate of oxygen uptake in a succinic dehydrogenase-cytochrome system, a systematic series of quantitative studies was made.

The experiments were carried out at 38° in Warburg Erlenmeyer flasks. The substrate, 0.3 cc. of 0.2 M succinic acid previously neutralized with NaOH, was added to the side arms. 0.1 cc. of cytochrome C and 1.0 cc. of 0.1 M phosphate buffer (pH 7.15) were added to each flask. Pure cytochrome C was prepared according to Keilin and Hartree (1) and was found by spectrophotometric analysis to contain 9.1×10^{-5} mM per cc. The total volume of reactants was always 3.3 cc. To prevent possible denaturation of the enzyme, the unbuffered aluminum chloride solution was always added last. The enzyme used was prepared by extracting washed minced beef heart muscle with alkaline phosphate according to a modification of the method of Stotz and Hastings (2) in which the alternate washings with warm water were omitted. In most cases this crude preparation was twice precipitated with 0.2 M acetate buffer of pH 4.5 and resuspended in phosphate buffer of pH 7.15. Unless otherwise specified, this will be the enzyme preparation referred to in all the following experiments.

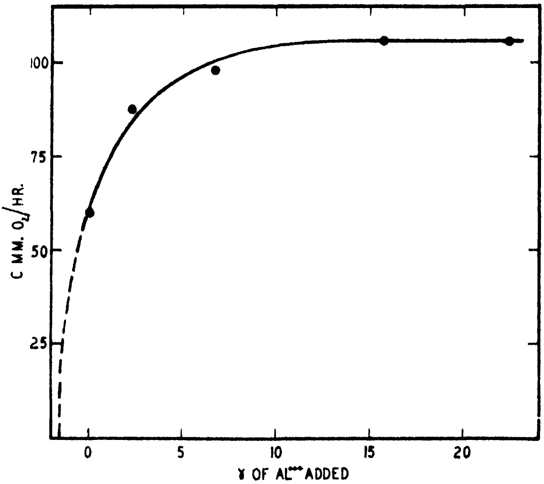


FIG. 1. The effect of increasing aluminum concentration on the rate of oxygen uptake of the succinic dehydrogenase-cytochrome C system. $T = 38°$, pH 7.15, succinate 0.06 mM total, cytochrome C 9.1×10^{-6} mM total, 0.1 cc. of enzyme.

With increasing concentrations of aluminum, the acceleration of succinate oxidation increased and approached a maximum value. A typical determination of this kind is shown in Fig. 1. By extrapolating the curve in Fig. 1 to zero activity (dotted line) it should be possible to estimate the aluminum concentration in the original enzyme preparation, assuming the aluminum was necessary to the system. This estimated value (1.5 micrograms per 0.1 cc.) was then compared with the spectrographically determined value for aluminum in an enzyme preparation and was found to be the same, within 50 per cent error. Chromium was found present in very much smaller concentrations, less than 0.10 microgram per 0.1 cc. We wish to express our appreciation to Professor W. C. Pierce for his cooperation in making these analyses.

Table I

Activation by Various Metal Ions

$T = 38°$, succinate 0.06 mM total, pH 7.15, cytochrome C 9.1×10^{-6} mM total, enzyme 0.1 cc.

Metal added	Amount of metal ion (total)	Velocity	Increase
		c.mm. O_2 per hr.	*per cent*
None	0.0	69.9	
$AlCl_3$	83.0 mM $\times 10^{-5}$	124.2	77.7
$CrCl_3$	40.0 " $\times 10^{-5}$	183.0	162.0
$NdCl_3$*	100 micrograms salt	123.9	77.4
$La(NO_3)_3$*	100 " "	149.4	114.0
$NaSm(SO_4)_2$*	100 " "	145.4	115.0
None	0.0	62.5	
$Mg(C_2H_3O_2)_2$	25.0 mM $\times 10^{-5}$	67.4	
$Mn(NO_3)_2$	20.0 " $\times 10^{-5}$	39.1	
$ZnSO_4$	20.0 " $\times 10^{-5}$	0.0	
None	0.0	113.5	
$FeCl_3$	40.0 mM $\times 10^{-5}$	99.0	

* The rare earth salts had a purity of 90 to 99 per cent. The impurities consisted of salts of other members of the rare earth group.

To determine whether aluminum was unique in possessing this accelerating property, the effect of the addition of various other salts was determined. As shown in Table I only the trivalent ions were effective. The exception in the case of ferric ion might be accounted for by the extreme insolubility of the hydroxide (pH of the reaction mixture = 7.15). Chromium was found to be more effective than aluminum, while zinc inhibited completely. A study with varying concentrations of chromium gave results similar to those obtained with aluminum (Fig. 1) except that chromium was in general about twice as active, mole for mole, and also gave a maximum which was twice that for aluminum. A very similar effect was observed by Lohmann and Schuster (3) and Ochoa and Peters (4) on the accelerating action of magnesium and manganese in the cocarboxylase system. Attempts to prepare an inactive preparation of succinic dehydrogenase which could be reactivated by adding aluminum were unsuccessful because of the inactivation of the enzyme by long dialysis.

Function of Aluminum

To determine whether the aluminum affected the cytochrome oxidase portion of the system or the succinic dehydrogenase, experiments were performed in such a way as to allow each of these parts to operate independently.

The physiological aerobic oxidation of succinate is now considered to be (*a*) the reduction of cytochrome C by succinate in the presence of succinic dehydrogenase, and (*b*) the oxidation of the reduced cytochrome by oxygen in the presence of cytochrome oxidase. Although Ball (5) has shown that cytochrome B under equilibrium conditions is 75 per cent reduced by succinic acid, this mechanism is probably of secondary importance to the cytochrome C system.

In a previous paper (6) it was shown that small concentrations of cyanide inhibit the aerobic oxidation of *p*-phenylenediamine to the extent of 85 to 90 per cent and that a very large concentration of cyanide is necessary for the complete inhibition. On the basis of Keilin's previous work (7) that the autoxidation of cytochrome B is not inhibited by small quantities of cyanide, it was assumed that the non-cyanide-sensitive portion of the system acting on *p*-phenylenediamine was very probably cytochrome B. In this investigation we have found that the aerobic oxidation of succinate is likewise inhibited only to the extent of 90 per cent by small concentrations of cyanide. In this respect the aerobic oxidation of *p*-phenylenediamine and that of succinate are very similar. Aluminum ion addition did not accelerate the "residual" aerobic oxidation of succinate after the addition of cyanide. We may therefore conclude that aluminum ion very probably does not affect the aerobic oxidation of succinate through cytochrome B.

Both *p*-phenylenediamine and hydroquinone are capable of rapidly reducing cytochrome C without the intervention of a specific dehydrogenase and, as substrates (with excess cytochrome), can be used as a measure of cytochrome oxidase activity (6). With systems in which these substrates are used aluminum gave no acceleration. We can therefore conclude that it does not affect the so defined cytochrome C oxidase portion of the system.

By inhibiting the action of cytochrome oxidase with cyanide, the succinic dehydrogenase activity can be tested with methylene blue as a hydrogen transporter. In this case too the addition of aluminum gave no acceleration, and we are forced to conclude that the aluminum does not affect this portion of the system as determined by this test.

When aluminum was added in large concentrations to the reaction mixture (lacking enzyme), a precipitate, presumably of aluminum hydroxide, was formed. With enzyme present this precipitate was much more voluminous, indicating adsorption or coprecipitation of some constituent. In the latter case the supernatant liquid obtained by low speed centrifugation was less active than the uncentrifuged suspension. Furthermore it was found that the precipitate itself was active. While no precipitate was observed with small aluminum ion addition, the natural turbidity due to the enzyme made the observation of such a precipitate very difficult.

We are inclined to believe that the explanation of the increased activity is in some way bound up with the precipitate formation and this action can only be satisfactorily interpreted after the succinic dehydrogenase-cytochrome system is better understood.

SUMMARY

1. The addition of aluminum ion to a succinic dehydrogenase-cytochrome oxidase system accelerates the rate of oxygen uptake. A limiting value of the acceleration is reached with very small amounts of aluminum.

2. Spectrographic analysis of the enzyme preparations indicated that the amount of aluminum found in these preparations was sufficient to account for the activity, assuming that aluminum is a necessary constituent.

3. Chromium and the rare earth salts produced the same effect but were found to be practically absent in a tissue extract.

4. The function of aluminum in succinate oxidation is discussed.

BIBLIOGRAPHY

1. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
2. Stotz, E., and Hastings, A. B., *J. Biol. Chem.*, **118**, 479 (1937).
3. Lohmann, K., and Schuster, P., *Biochem. Z.*, **294**, 188 (1937).
4. Ochoa, S., and Peters, R. A., *Biochem. J.*, **32**, 150 (1938).
5. Ball, E. G., *Biochem. Z.*, **295**, 262 (1938).
6. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., *J. Biol. Chem.*, **124**, 733 (1938).
7. Keilin, D., *Proc. Roy. Soc. London, Series B*, **104**, 206 (1929).

THE CHEMICAL ACTIVATION OF STEROLS

V. A STUDY OF THE RELATIONSHIP BETWEEN CHEMICAL ACTIVATION AND CONFIGURATION OF VARIOUS STEROLS AND DERIVATIVES*

By JOHN C. ECK and BYRON H. THOMAS

(From the Animal Chemistry and Nutrition Subsection, Agricultural Experiment Station, Iowa State College, Ames)

(Received for publication, January 13, 1939)

It has previously been shown (1) that cholesterol and various cholesterol derivatives such as cholesterilene, dicholesteryl ether, cholesteryl chloride, cholestene, and butyl cholesteryl ether yield antirachitic products when treated with the sulfuric acid-acetic anhydride reagent, whereas cholestenone and dihydrocholesterol do not. These results pointed to a specific structural relationship which appeared to be essential to the chemical antirachitic activation of cholesterol and cholesterol derivatives. The probability of this relationship between chemical activation and configuration has been investigated further by testing the reaction products obtained by the action of the sulfuric acid-acetic anhydride reagent on a number of sterols and additional cholesterol derivatives for antirachitic activity.

Preparation of Sterols and Derivatives

Cholestane, m.p. 79-80°, and coprostane, m.p. 67-68°, were obtained by the catalytic (PtO_2) hydrogenation of cholestene (2) and pseudocholestene (3) respectively. They were purified in carbon tetrachloride solution by the action of acetic anhydride and concentrated sulfuric acid according to the method of Anderson and Nabenhauer (4) and then recrystallized from ether-alcohol. Neocholestene, m.p. 75-76°, was prepared by the action of quinoline on α-cholestyl chloride, obtained by the action of thionyl

---

* Journal Paper No. J606 of the Iowa Agricultural Experiment Station, Project No. 506.

chloride on dihydrocholesterol purified as its acetate (4), according to the method of Mauthner (3), and purified by bromination followed by subsequent debromination and recrystallization according to the method of Hattori (5). Pseudocholestene, m.p. 78–79°, was prepared by the action of alcoholic potassium acetate on cholestene hydrochloride (5-chlorocholestane) according to the method of Mauthner and Suida (6). 4,6-Cholestadiene, m.p. 84–85°, and 7-dehydrocholestene isomer, m.p. 91–92°, were prepared by the action of quinoline on β-cholestene dibromide (5,6-dibromocholestane) and by the action of alcoholic hydrochloric acid on 7-hydroxycholestene respectively (7). 7-Dehydrocholestene, m.p. 88–89°, was obtained by the vacuum distillation of the benzoate of 7-hydrocholestene according to the method of Dimroth and Trautmann (8).

Allocholesterol, m.p. 131–132°, and epiallocholesterol, m.p. 83–84°, were obtained by the reduction of cholestenone with aluminum isopropoxide, followed by separation of the two isomers with digitonin according to the method of Schoenheimer and Evans (9). Pseudocholesterol, m.p. 115–116°, was prepared by the sodium-alcohol reduction of 7-ketocholesterilene according to the method of Windaus and Resau (10). 7-Hydroxycholestene, m.p. 93–94°, was obtained by the aluminum isopropoxide reduction of 7-ketocholestene according to the method of Dimroth and Trautmann (8).

7-Ketocholestene, m.p. 124–125°, 7-ketocholesteryl acetate, m.p. 153–154°, and 7-ketocholesteryl chloride, m.p. 121–122°, were prepared from the neutral oxidation products obtained by the chromium trioxide oxidation of cholestene (11), cholesteryl acetate (12), and cholesteryl chloride (13) respectively and were purified by recrystallization from alcohol. 7-Ketocholesterilene, m.p. 91–92°, was prepared by the action of alcoholic hydrochloric acid on 7-ketocholesteryl acetate according to the method of Stavely and Bergmann (14). Cholestanone, m.p. 142–143°, was obtained by the chromium trioxide oxidation of dihydrocholesterol (15).

i-Cholesterol, m.p. 74–75°, was obtained by the action of an aqueous acetone solution of potassium acetate on cholesteryl *p*-toluenesulfonate according to the method of Beynon, Heilbron, and Spring (16), followed by saponification and treatment with digitonin. *a*-Cholesterilene, m.p. 344° (bloc Maquenne), and *c*-cholesterilene, m.p. 200° (bloc Maquenne), were obtained by the action of sulfuric acid on cholesterol according to a modification (17) of the method of Zwenger (18). The hydrocarbon $C_{26}H_{44}$, described as the hydrocarbon of melting point 67° by Lettré (11), m.p. 67°, was prepared by the distillation of the acid fraction of the chromium trioxide oxidation of cholestene (11) and was purified by recrystallization from ether-alcohol. The hydrocarbon $C_{26}H_{42}$, described as the hydrocarbon $C_{25}H_{40}$ by Windaus and Resau (10) and as the diethenoid hydrocarbon by Barr, Heilbron, Parry, and Spring (19), m.p. 75.5–76°, was obtained by the distillation of the acid fraction of the chromium trioxide oxidation of cholesteryl acetate (12) and was purified by recrystallization from ether-alcohol. The compound $C_{19}H_{30}O$, described as 13,18-dimethyl-9,13-cyclopentano-5,6-dehydrohydrophenanthr-3-ol by de Fazi and Pirrone (20), m.p. 202–203°, was obtained by the action of cuprous chloride on cholesterol (20) and was purified by recrystallization from alcohol.

Commercial ergosterol was purified according to the method of Bills and Honeywell (21) until it melted at 162–163° and $[\alpha]_D^{25}$ was $-130.6°$ (*c*, 2.99 in CCl_4). Stigmasterol, m.p. 170–171°, and $[\alpha]_D^{25} = -44.9°$ (*c*, 3.01 in CCl_4), was obtained by saponification of soy bean oil according to the method of Steiger and Reichstein (22). *β*-Sitosterol (tall oil sitosterol), m.p. 138–139°, $[\alpha]_D^{25} = -31.9°$ (*c*, 3.00 in CCl_4), was obtained by the saponification of pine oil according to the method of Sandqvist and Bengtsson (23). Lanosterol, m.p. 140–141° and $[\alpha]_D^{25} = +57.7°$ (*c*, 2.98 in CCl_4), was obtained from the unsaponifiable fraction of wool fat according to the method of Lifschütz and Vietmeyer (24) and purified according to the method of Windaus and Tschesche (25).

Results of Treatment with Reagent

The various sterols and cholesterol derivatives were treated with the sulfuric acid-acetic anhydride reagent according to the procedure previously described (1). Each compound was heated in a 50 cc. Erlenmeyer flask at 85–90° for 3 hours with 0.002 mole of concentrated sulfuric acid and 0.0025 mole of acetic anhydride in 4 cc. of glacial acetic acid per 0.001 mole of compound treated. The average healing response of groups of rachitic rats to the various reaction products obtained is presented in Table I, according to test number. Several of the reaction products failed to produce any macroscopic evidence of calcification in the rachitic metaphyses of rats fed the supplement at the levels indicated. It

TABLE I

Effect of Sulfuric Acid-Acetic Anhydride in Imparting Antirachitic Activity to Various Sterols and Cholesterol Derivatives

Test No.	Compound treated	Level*	Line test† (average + values)
1	Cholestane	31.10	0
2	Coprostane	31.10	0
3	Neocholestene	31.10	0
4	Pseudocholestene	1.56	2.0
5	4,6-Cholestadiene	3.11	2.0
6	7-Dehydrocholestene isomer	1.56	2.5
7	7-Dehydrocholestene	1.56	2.0
8	Allocholesterol	1.56	2.5
9	Epiallocholesterol	1.56	2.0
10	Pseudocholesterol	3.11	2.0
11	7-Hydroxycholestene	1.56	2.0
12	Cholesterol	1.56	2.5
13	7-Ketocholestene	31.10	0
14	7-Ketocholesteryl acetate	31.10	0
15	" chloride	31.10	0
16	7-Ketocholesterilene	31.10	0
17	Cholestanone	31.10	0
18	*i*-Cholesterol	3.11	2.5
19	*a*-Cholesterilene	31.10	0
20	*c*-Cholesterilene	31.10	0
21	Hydrocarbon $C_{26}H_{44}$	31.10	0
22	" $C_{26}H_{42}$	31.10	0
23	Compound $C_{19}H_{30}O$	3.11	1.5
24	Ergosterol	31.10	2.0
25	Stigmasterol	31.10	2.0
26	β-Sitosterol	31.10	2.5
27	Lanosterol	31.10	0

* Per cent of the reaction product obtained per 0.001 mole of the original compound fed per rat; thus 1.56, 3.11, and 31.10 per cent of the reaction products obtained from 0.001 mole of original compound are equivalent on the molar basis to the amounts of reaction product obtained from 6, 12, and 120 mg. of cholesterol respectively.

† A 2 + value denotes approximately 5 vitamin units, U. S. P. XI (26). The tests were conducted with one or more groups of six rats each.

is doubtful whether healing can be produced by feeding these reaction products in larger quantities.

The reaction mixtures containing cholestane and coprostane remained colorless and the reaction products from these two derivatives were found to be inactive (Tests 1 and 2); unchanged cholestane and coprostane were recovered. The reaction mixture containing neocholestene became violet in color and the reaction product was found to be inactive (Test 3). The reaction mixtures containing pseudocholestene, 4,6-cholestadiene, 7-dehydrocholestene isomer, 7-dehydrocholestene, allocholesterol, epiallocholesterol, pseudocholesterol, 7-hydroxycholestene, and cholesterol underwent the same green to brown color change and the reaction products were found to be active (Tests 4 to 12).

The reaction mixtures containing 7-ketocholestene, 7-ketocholesteryl acetate, 7-ketocholesteryl chloride, 7-ketocholesterilene, and cholestanone became brown in color and the reaction products were found to be inactive (Tests 13 to 17). The reaction mixture containing *i*-cholesterol changed through green to brown in color and the reaction product was found to be active (Test 18); *a*-cholesterilene was isolated from the reaction product. The reaction mixtures containing *a*- and *c*-cholesterilenes became brown in color and the reaction products were found to be inactive (Tests 19 and 20). The reaction mixtures containing the hydrocarbons $C_{26}H_{44}$ and $C_{26}H_{42}$ became red to brown in color and both reaction products are found to be inactive (Tests 21 and 22). The reaction mixtures containing the compound $C_{19}H_{30}O$ became green to brown in color and the reaction product was found to be active (Test 23).

The reaction mixtures containing ergosterol, stigmasterol, and β-sitosterol became green to brown in color and the reaction products were found to be active (Tests 24 to 26). The reaction mixture containing lanosterol became brown in color and the reaction product was found to be inactive (Test 27).

DISCUSSION

From the results obtained by biologically testing the reaction products produced by the action of the sulfuric acid-acetic anhydride reagent on various sterols and cholesterol derivatives, additional evidence was found in support of the previously reported suggestion of a specific relationship between chemical antirachitic activation and configuration. Cholesterol derivatives such as cholestane and coprostane which have no functional groups were found to yield inactive products. Neocholestene (cholestene-2) yielded an inactive product, whereas that obtained from pseudocholestene (cholestene-4) was active. Cholestadienes such as 4,6-cholestadiene, 7-dehydrocholestene isomer (the structure of which is unknown (7)), and 7-dehydrocholestene and cholestenols such as allocholesterol (cholesten-4-ol-3), epiallocholesterol (cholesten-4-ol-3-epi), 7-hydroxycholestene (cholesten-5-ol-7), and cholesterol (cholesten-5-ol-3) yielded active products. Thus several cholesterol derivatives having a double bond in the 4- or 5-position and with or without an additional double bond or hydroxyl group have been found to yield antirachitic products by the action of the sulfuric acid-acetic anhydride reagent.

Antirachitic products have been obtained (1) by the action of the sulfuric acid-acetic anhydride reagent on cholestene (cholestene-5), cholesteryl acetate (cholesten-5-ol-3 acetate), cholesteryl chloride (3-chlorocholestene-5), and cholesterilene (3,5-cholestadiene (14, 17)). On the other hand, inactive products were produced by the action of the same reagent on the 7-keto derivatives of these cholesterol derivatives; namely, 7-ketocholestene (cholesten-5-one-7), 7-ketocholesteryl acetate (cholesten-5-one-7-ol-3 acetate), 7-ketocholesteryl chloride (3-chlorocholesten-5-one-7), and 7-ketocholesterilene (cholestadiene-3,5-one-7). An antirachitic product was also obtained from pseudocholestene, whereas cholestenone (cholesten-4-one-3) has been found to yield an inactive product (1). Inactive products were obtained from both dihydrocholesterol (cholestanol-3) (1) and its ketone derivative cholestanone (cholestanone-3). Although several cholesterol derivatives having a double bond in the 4- or 5-position have been converted into active products, the 3- and 7-keto derivatives of these compounds were not converted into active products. The change of an adjacent methylene group into a carbonyl group (either 3- or 7-position) converts the compounds from olefines into α,β-unsaturated ketones which would stabilize the double bonds in the 4- or 5-position. *i*-Cholesterol, which is a molecular rearrangement product of cholesterol (16, 27–29) was found to yield an active product. *a*- and *c*-cholesterilenes, the structures of which are unknown (17), were found to yield inactive products. The hydrocarbon $C_{26}H_{44}$, which differs from cholestene in that Ring B is 5-membered so that the double bond may be described as between carbon atoms 5 and 7, was found to yield an inactive product. The hydrocarbon $C_{26}H_{42}$, which has the same structure as hydrocarbon $C_{26}H_{44}$ with an additional double bond between carbon atoms 3 and 4 (9, 19), was found to yield an inactive product by the action of the reagent. The hydrocarbons $C_{26}H_{44}$ and $C_{26}H_{42}$ do not possess the cyclopentanoperhydrophenanthrene nucleus. The compound $C_{19}H_{30}O$ which has been described by de Fazi and Pirrone (20) to have the same structure as cholesterol with the exception of a hydrogen atom in place of the isooctyl side chain on carbon atom 17 was found to yield an active product by the action of the reagent.

Ergosterol, stigmasterol, and β-sitosterol (22-dihydrostigmasterol (30-32)) were found to yield products possessing slight antirachitic activity by the action of the sulfuric acid-acetic anhydride reagent. These sterols differ from cholesterol by additional double bonds in the 7- and 22-positions and a methyl group in place of a hydrogen atom in the 24-position, by an additional double bond in the 22-position and an ethyl group in place of a hydrogen atom in the 24-position, and by an ethyl group in place of a hydrogen atom in the 24-position respectively. Lanosterol, a triterpene (33), was found to yield an antirachitically inactive product by the action of the reagent.

SUMMARY

The sulfuric acid-acetic anhydride reagent was found to be effective in converting pseudocholestene, 4,6-cholestadiene, 7-dehydrocholestene isomer, 7-dehydrocholestene, allocholesterol, epiallocholesterol, pseudocholesterol, *i*-cholesterol, and compound $C_{19}H_{30}O$ into active products. This reagent was also found to convert ergosterol, stigmasterol, and β-sitosterol into less active products. Antirachitically inactive products were produced by the action of the sulfuric acid-acetic anhydride reagent on cholestane, coprostane, neocholestene, 7-ketocholestene, 7-ketocholesteryl acetate, 7-ketocholesteryl chloride, 7-ketocholesterilene, cholestanone-3, hydrocarbon $C_{26}H_{44}$, hydrocarbon $C_{26}H_{42}$, *a*-cholesterilene, *c*-cholesterilene, and lanosterol.

The various sterols or derivatives treated which possessed the cyclopentanoperhydrophenanthrene nucleus, having a double bond in the 4- or 5-position, with or without an additional double bond or hydroxyl group, and without a ketone group in the 3- or 7-position, were converted into antirachitic products by the action of the sulfuric acid-acetic anhydride reagent.

BIBLIOGRAPHY

1. Eck, J. C., Thomas, B. H., and Yoder, L., *J. Biol. Chem.*, **117**, 655 (1937).
2. von Furth, O., and Felsenreich, G., *Biochem. Z.*, **69**, 416 (1915).
3. Mauthner, J., *Monatsh. Chem.*, **30**, 635 (1909).
4. Anderson, R. J., and Nabenhauer, F. P., *J. Am. Chem. Soc.*, **46**, 1957 (1924).
5. Hattori, K., *J. Pharm. Soc. Japan*, **57**, 708 (1937).
6. Mauthner, J., and Suida, W., *Monatsh. Chem.*, **28**, 1113 (1907).
7. Eck, J. C., Van Peursem, R. L., and Hollingsworth, E. W., *J. Am. Chem. Soc.*, **61**, 171 (1939).
8. Dimroth, K., and Trautmann, G., *Ber. chem. Ges.*, **69**, 669 (1936).
9. Schoenheimer, R., and Evans, E. A., Jr, *J. Biol. Chem.*, **114**, 567 (1936).
10. Windaus, A., and Resau, C., *Ber. chem. Ges.*, **48**, 851 (1915).
11. Lettré, H., *Z. physiol. Chem.*, **218**, 67 (1933).
12. Windaus, A., Lettré, H., and Schenck, F., *Ann. Chem.*, **520**, 98 (1935).
13. Mauthner, J., and Suida, W., *Monatsh. Chem.*, **17**, 579 (1896).
14. Stavely, H. E., and Bergmann, W., *J. Organ. Chem.*, **1**, 567 (1937).
15. Bruce, W. F., in Fieser, L. F., Organic syntheses, **17**, New York, 43 (1937).
16. Beynon, J. H., Heilbron, I. M., and Spring, F. S., *J. Chem. Soc.*, 1459 (1937).
17. Eck, J. C., and Van Peursem, R. L., *Iowa State Coll. J. Sc.*, **13**, 115 (1939).
18. Zwenger, C., *Ann. Chem.*, **66**, 5 (1848).
19. Barr, T., Heilbron, I. M., Parry, E. G., and Spring, F. S., *J. Chem. Soc.*, 1437 (1936).
20. de Fazi, R., and Pirrone, F., *Atti. accad. Lincei, Classe sc. fis., mat., e nat.*, **23**, 887 (1936).
21. Bills, C. E., and Honeywell, E. M., *J. Biol. Chem.*, **80**, 15 (1928).
22. Steiger, M., and Reichstein, T., *Helv. chim. acta*, **20**, 1040 (1937).
23. Sandqvist, H., and Bengtsson, E., *Ber. chem. Ges.*, **64**, 2167 (1931).
24. Lifschütz, I., and Vietmeyer, O., *Z. physiol. Chem.*, **115**, 241 (1926).
25. Windaus, A., and Tschesche, R., *Z. physiol. Chem.*, **190**, 51 (1930).
26. The pharmacopœia of the United States of America, eleventh decennial revision, Easton, 478–482 (1936).

27. Wallis, E. S., Fernholz, E., and Gephart, F. T., *J. Am. Chem. Soc.*, **59**, 137 (1937).
28. Ford, E. G., and Wallis, E. S., *J. Am. Chem. Soc.*, **59**, 1415 (1937).
29. Ford, E. G., Chakovarty, P., and Wallis, E. S., *J. Am. Chem. Soc.*, **60**, 413 (1938).
30. Bengtsson, B. E., *Z. physiol. Chem.*, **237**, 46 (1935).
31. Bernstein, S., and Wallis, E. S., *J. Organ. Chem.*, **2**, 341 (1937).
32. Marker, R. E., and Wittle, E. L., *J. Am. Chem. Soc.*, **59**, 2704 (1937).
33. Schulze, H., *Z. physiol. Chem.*, **238**, 35 (1936).

THE CHEMICAL ACTIVATION OF STEROLS

VI. A STUDY OF VARIOUS REAGENTS IN CHEMICAL ACTIVATION AND IN STEROL COLOR REACTIONS*

By JOHN C. ECK and BYRON H. THOMAS

(From the Animal Chemistry and Nutrition Subsection, Agricultural Experiment Station, Iowa State College, Ames)

(Received for publication, January 13, 1939)

In the investigation of the chemical antirachitic activation of sterols and cholesterol derivatives, it has been shown that various acidic dehydrating reagents are effective in the production of antirachitic products (1, 2) and that the compounds treated (3, 4) which possessed the cyclopentanoperhydrophenanthrene nucleus with a double bond in the 4- or 5-position, with or without an additional double bond or hydroxyl group, and without a carbonyl group in the 3- or 7-position, were converted into antirachitic products by the action of sulfuric acid in the presence of acetic anhydride. Temperature, time, proportion of the reagent (1), and the anhydrous conditions of the reaction mixture (2) were found to be important in the reactions employed in chemical activation.

Some of the reagents used in chemical activation, namely sulfuric acid, sulfuric acid with acetic anhydride, zinc chloride, and trichloroacetic acid, are employed in sterol color reactions under different conditions of temperature, time, and solvent used. The treatment of cholesterol and cholesterol derivatives with various reagents used in sterol color reactions was studied in order to correlate chemical activation with color reactions.

EXPERIMENTAL

The numerous sterol color reactions were reviewed and the following experimental work was conducted in order to provide

---

* Journal Paper No. J612 of the Iowa Agricultural Experiment Station, Project No. 506.

TABLE I

Effect of Sulfuric Acid in Imparting Antirachitic Activity to Cholesterol and Several Cholesterol Derivatives

Reaction No.	Compound treated*	Reagent used	Amount	Time	Color change observed†	Level‡	Line test§ (average + values)
			0.001 mole	*hrs.*			
1	Cholesterol	H_2SO_4	2.0	3	*v-g-bp*	3.11	2.5
2	Cholesterilene	"	2.0	3	*bc-sb-pr*	3.11	2.0
3	Cholestene	"	2.0	3	*t-g*	31.10	2.5
4	Cholesteryl acetate	"	2.0	3	*v-g-p*	3.11	2.0
5	" chloride	"	2.0	3	*v-g*	3.11	3.0
6	" butyl ether	"	2.0	3	*g-b*	3.11	2.5
7	Dicholesteryl ether	"	4.0	3	*b-g*	3.11	2.5
8	Cholestene	"	4.0	3	"	20.7	2.5
9	Cholesterol	H_2SO_4 + AcCl	2.0 2.5	3	*g-b*	1.56	2.0
10	"	H_2SO_4 + Ac_2O	2.0 2.5	3	*g-b*	1.56	2.5
11	"	H_2SO_4 + Ac_2O	2.0 2.5	192	*g-b*	12.44	0
12		Then heated		3		3.11	2.0
13	"	H_2SO_4 + Ac_2O with irradiation	2.0 2.5	0.5	*g-b*	1.56	2.5

14	Cholesterol	H_2SO_4 + $(HCHO)_3$	2.0 1.0	3	*a–v–g–bp*	3.11	2.5
15	"	Bz_2O_2 then H_2SO_4	1.0 2.0	1 3	*ap–g–bp*	3.11	2.5

---

[*] The compounds, 0.001 mole each except 0.0005 mole in the case of Reaction 7, dissolved in 4 cc., except 8 cc. in the case of Reactions 7 and 8, of glacial acetic acid were heated with the reagents at 85–90°, except 25° in the case of Reaction 11.

[†] Color legend, *a* blue, *b* brown, *c* pink, *g* green, *p* purple, *r* red, *s* gray, *t* light brown, and *v* violet.

[‡] Per cent of the reaction product obtained per 0.001 mole of compound treated except 0.0005 mole in the case of Reaction 7 (4).

[§] A 2 + value denotes approximately 5 vitamin D units, U.S.P. XI (5). The tests were conducted with one or more groups of six rats each.

TABLE II

Effect of Various Reagents in Imparting Antirachitic Activity to Cholesterol, Cholesterilene, and Cholestene

Reaction No.	Compound treated	Amount	Reagent used	Amount	Solvent used	Amount	Temperature	Time	Color change observed*	Quantity of product fed per rat	Line test† (average + values)
		0.001 mole		*0.001 mole*		*cc.*	*°C.*	*hrs.*		*mg.*	
16	Cholesterol	4	$ZnCl_2$	14.6	HOAc	16	85–90	3	*c–d–e*	400	3.0
17	"	4	"	14.6	"	16	85–90	3	*ad–e*	400	3.0
			+ $(HCHO)_3$	4.0							
18	"	4	$ZnCl_2$	14.6	HOAc	16	85–90	3	*ad–e*	400	3.5
			+ Bz_2O_2	4.0							
19	"	2	$ZnCl_2$	7.3	HOAc	8	85–90	3	*c–d–r–b*	6	1.5
			+ AcCl	5.0							
20	"	2	$ZnCl_2$	7.3	HOAc	8	85–90	3	*c–r–p–b*	6	2.0
			+ BzCl	5.0							
21	"	2	$ZnCl_2$	7.3	HOAc	8	85–90	3	*c–t*	24	1.0
			+ Ac_2O	5.0							
22	"	2	$ZnCl_2$	7.3	HOAc	8	85–90	3	*c–d–r–b*	12	1.5
			+ Ac_2O	5.0							
			+ HCl	Excess							
23	Cholesterilene	2	$ZnCl_2$	7.3	HOAc	8	85–90	3	*t–bp–b*	6	2.5
			+ AcCl	5.0							
24	Cholestene	4	$ZnCl_2$	14.6	HOAc	16	85–90	3	*d–bg*	400	2.5
			+ AcCl	10.0							
25	Cholesterol	7.78	CCl_3COOH	7.78			160	0.166	*r–t*	400	3.5
26	Cholesterilene	6.8	"	48.2			25	6	*v–p–bp*	250	0

27	Cholesterilene	2.72	CCl_3COOH	19.3			160	0.166	*p–bp*	150	2.5
28	Cholestene	6.8	"	48.2			160	0.166	*r–b*	400	2.0
29	Cholesterol	8.1	HCl	Excess	Alcohol	45	25	72	*y–t–rbg*‡	400	Trace?
					Ether	45					
30	Cholesterilene	2.7	"	"	Alcohol	15	25	72	*y–t–rbg*	75	2.5
					Ether	15					
31	Cholestene	16.2	"	"	Alcohol	90	25	72	*t*	400	0
					Ether	90					
32	Cholesterol	5	P_2O_5	7	C_6H_6	40	80	2	*p*	300	2
33	Cholesterilene	5	"	7	"	40	80	2	"	300	3
34	Cholestene	5	"	7	"	40	80	2	*y*	300	0

* Color legend, *a* blue, *b* brown, *c* pink, *d* rose, *e* buff, *g* green fluorescence, *p* purple, *r* red, *t* light brown, *v* violet, and *y* yellow.

† A 2 + value here denotes approximately 5 vitamin D units, U.S.P. XI (5). The tests were conducted with one or more groups of six rats each.

‡ Slow color change.

data for a discussion of chemical activation in connection with sterol color reactions. Cholesterol and a number of cholesterol derivatives were treated with various reagents used in sterol color reactions. The experimental conditions employed and the biological tests of the reaction products obtained are presented in Tables I and II. The production of *a*-cholesterilene as a by-product of some of these reactions is described elsewhere (6).

The description of the reactions concerning sulfuric acid are presented in Table I according to reaction number, and that of the reactions involving zinc chloride, trichloroacetic acid, hydrogen chloride, and phosphorus pentoxide are presented in Table II. The aqueous washings of the reaction products obtained by the action of trichloroacetic acid were found to give negative tests for chloride ion. Reaction products which possessed definite antirachitic activity at the levels tested were not obtained with other reagents such as antimony trichloride, antimony pentachloride, arsenic trichloride, or nitric acid.

The following compounds give a positive Liebermann-Burchard color reaction through rose-red to violet to blue to green: cholesterol, cholesterilene, cholestene, cholesteryl acetate, cholesteryl chloride, butyl cholesteryl ether, dicholesteryl ether, pseudocholestene, 4,6-cholestadiene, 7-dehydrocholestene, 7-dehydrocholestene isomer, allocholesterol, epiallocholesterol, pseudocholesterol, 7-hydroxycholestene, *i*-cholesterol, compound $C_{19}H_{30}O$, ergosterol, stigmasterol, and *β*-sitosterol. The following compounds give a negative Liebermann-Burchard color reaction with the display of a brown coloration unless otherwise stated: dihydrocholesterol (colorless), cholestenone, cholestane (colorless), coprostane (colorless), neocholestene (violet), 7-ketocholestene, 7-ketocholesteryl acetate, 7-ketocholesteryl chloride,7-ketocholesterilene, cholestanone, *a*-cholesterilene, *c*-cholesterilene, hydrocarbon $C_{26}H_{44}$ (cherry-red to blue-violet), hydrocarbon $C_{26}H_{42}$ (cherry-red to blue-violet), and lanosterol. Treated with zinc chloride and acetyl chloride in acetic acid as in the Tschugajeff reaction but at room temperature, cholesterilene gives a rapid color change through red to purple, cholesterol gives a slow color change through pink to red to purple, and cholestene gives a weak color change through pink to red.

DISCUSSION

Sulfuric acid is used at room temperature without dilution in the Salkowski reaction (7–9) and in the Drummond and Watson reaction (10, 11), diluted to a specific gravity of 1.76 in the Hesse reaction (9, 12), diluted to 10 M in a modified Moleschott reaction (13), with a sugar in the Neuberg reaction (14), with acetic anhydride without a solvent in the Liebermann reaction (15), with acetic anhydride in chloroform solution in the Liebermann-Burchard reaction (16), with formaldehyde in the Whitby A and B reactions (9, 10), and following pretreatment with benzoyl peroxide in the Lifschütz reaction (9, 17). A more intense color reaction is obtained with sulfuric acid when concentrated than when diluted and also when in the presence of acetic anhydride or formaldehyde or following pretreatment with benzoyl peroxide than with sulfuric acid alone. In chemical activation it has been found that heat is required for all activating reagents used except hydrogen chloride (1, 2) and that maximum activity is obtained when the reaction mixture is anhydrous (2). Both acetyl chloride (Reaction 9, Table I) and acetic anhydride (Reaction 10) appear to have increased the activity of the reaction product obtained by the treatment of cholesterol with sulfuric acid, whereas neither paraformaldehyde (Reaction 14) nor benzoyl peroxide (Reaction 15) has this apparent effect. It was found that the compounds (3, 4) which yielded active products by the action of sulfuric acid with acetic anhydride gave a positive Liebermann-Burchard color reaction, whereas the compounds which did not yield active products do not give a positive color test. A variety of other compounds has been tested with the Liebermann-Burchard color reaction and different colors or color changes have been observed (18). Heat as well as the color change is of importance in chemical activation by means of sulfuric acid and acetic anhydride, since the same color change was observed at room temperature (Reaction 11) as when heated (Reaction 25, Table II), although the product obtained at room temperature (Reaction 11) was inactive and was converted into an active product when subsequently heated (Reaction 12). The color change observed and the activation produced were effected in less time when irradiated during the reaction (Reaction 13), since a period of 3 hours was found necessary (2) for maximum activation without irradiation.

An eosin red color develops when an acetic acid solution of cholesterol is heated with zinc chloride and acetyl chloride or benzoyl chloride in the Tschugajeff reaction (19). A purple color is obtained when cholesterol is heated with zinc chloride alone in acetic acid solution and the presence of formaldehyde intensifies the purple color (9). Cholesterilene and cholestene also give a positive Tschugajeff reaction (10). At room temperature the Tschugajeff reaction is more intense with cholesterilene and less intense with cholestene than with cholesterol and the activity of the reaction products obtained when heated was apparently greater with cholesterilene (Reaction 23), and less with cholestene (Reaction 24) than with cholesterol (Reaction 19). It is evident (Reactions 16, 21, 22) that the zinc chloride, the anhydrous state of the reaction medium due to the acetyl chloride, and the hydrogen chloride formed upon hydrolysis of the acetyl chloride by water present in the acetic acid are probably all of importance in the activation of cholesterol by the action of zinc chloride with acetyl chloride (Reaction 19). The color reaction with this reagent in acetic acid is thus parallel with the chemical activation of cholesterol, cholesterilene, and cholestene, although the display of color in the activation of cholesterol with zinc chloride is not necessary in the production of an active product, since an active product was produced by the treatment of cholesterol with zinc chloride in the absence of a solvent without the display of a red color (1).

Blue colorations without material increases in activity of the reaction products were obtained by the presence of paraformaldehyde (Reaction 17) or of benzoyl peroxide (Reaction 18) during the treatment of cholesterol with zinc chloride in acetic acid. A blue coloration is also involved under conditions which may be inferred as oxidative in the color reactions of antimony and arsenic trichlorides which yielded products possessing no definite antirachitic activity. Thus antimony trichloride does not give a characteristic color with cholesterol (20-23) in the Carr and Price reaction (24) but does give a blue coloration with cholesterol which had been heated in the air (25), heated in colloidal aqueous suspension (25), irradiated in the presence of oxygen (22), or following treatment with benzoyl peroxide or formaldehyde (26) and with 7-hydroxy sterol derivatives (27). Arsenic trichloride gives a red color with cholesterol (21, 28) in the Rosenheim and Drummond reaction (29) and blue colorations with cholesterol which had been pretreated with benzoyl peroxide or formaldehyde (30).

An intimate mixture of cholesterilene and 90 per cent trichloroacetic acid displays a red color at room temperature in the Rosenheim reaction (31), whereas cholesterol and cholestene do not (32). The Rosenheim color reaction has been found to be positive for sterols and sterol derivatives containing or potentially containing a conjugated system of double bonds (32). When heated with freshly distilled trichloroacetic acid, color changes through red or purple to brown with the formation of active products were obtained with cholesterol (1), cholesterilene (Reaction 26) and cholestene (Reaction 27) but at room temperature the color change was observed with the formation of an inactive product from cholesterilene (Reaction 28). When heated, the color reaction observed with trichloroacetic acid was apparently correlative with the chemical activation of cholesterol, cholesterilene, and cholestene. The formation of chloride ions is involved in the treatment of cholesterol but not cholesterilene or cholestene with a quantity of trichloroacetic acid, which is probably a dehydrating reagent, in larger (previously described (1)) than equimolar (Reaction 25) proportion.

A yellow to light brown to reddish brown color with a green fluorescence was observed with the formation of an active product by the action of hydrogen chloride in ether on cholesterilene but not in the case of cholesterol or cholestene (1). Ether-alcohol was found to be a better solvent than ether for the display of color change and for chemical activation with this reagent (Reactions 29 to 31).

It has been reported (9) that when a chloroform solution of the hydrocarbons (not characterized) obtained by the treatment of cholesterol with sulfuric acid or zinc chloride was warmed with phosphorus pentoxide, the reaction mixture displayed a rose to cherry-red color change. Furthermore a purple color developed when the solution was pretreated with formaldehyde, and a color change through blue to green was observed when the purple solution was poured off and treated with acetic anhydride. A mixture of phosphorus pentoxide and cholesterol or cholesterilene heated in the absence of a solvent was observed to display a purple to brown color change with the formation of an active product (1). Activation with phosphorus pentoxide in benzene solution was found correlative with the (purple) color display in the treatment of cholesterol (Reaction 32), cholesterilene (Reaction 33), and cholestene (Reaction 34).

Other reagents used in sterol color reactions include antimony pentachloride and nitric acid which yielded reaction products possessing no definite antirachitic activity. Antimony pentachloride in the Steinle and Kahlenberg color reaction gives a brown complex, $C_{27}H_{45}OH \cdot SbCl_5$, with cholesterol which when dissolved in different solvents displays various colors (20). In the Whitby C color reaction cholesterol which has been preheated with acetic anhydride gives a blue or blue-green color when treated with nitric acid (9). In the Rosenheim and Callow color reaction, a chloroform solution of a sterol displays a color when treated with nitric acid and mercuric acetate (33). Color reactions have been observed for various sterols or sterol derivatives by treatment with additional reagents such as hydrogen iodide containing iodine (34), bromine in chloroform solution in the Tortelli-Jaffe reaction (35), chloral hydrate (31), silicotungstic acid (36), rosaniline (37), benzidine (37), selenic acid (38), dimethyl sulfate (29), and floridin (39). Cholesteryl propionate displays a series of color changes when cooled after fusion in the Obermüller reaction (9, 40).

The red color in the Salkowski reaction has been suggested as due to cholesteryl acid sulfate (8) and the green color in the Liebermann-Burchard reaction to cholesteryl esters (41) but cholesteryl acid sulfate in the form of its potassium or ammonium salts was found to be antirachitically inactive (1). Sulfuric acid (40), antimony trichloride (40), and antimony pentachloride (20) have been reported to form colored addition compounds with cholesterol at room temperature. The action of formaldehyde, benzoyl peroxide, and nitric acid may be inferred as oxidative in influence in various color reactions. Experimental data do not indicate that addition compound formation or oxidative influences are involved in chemical activation. It was found (2) that the sulfur dioxide evolved as an oxidative side reaction during the action of sulfuric acid and acetic anhydride on cholesterol was not correlative with the activity produced.

The reaction products obtained by the action of sulfuric acid on cholestene (Reactions 3, 8) were less active than those obtained from cholesterol or certain other cholesterol derivatives (Reactions 1, 2, 4 to 7). The reaction products produced by means of zinc chloride and acetyl chloride, trichloroacetic acid, hydrogen chloride, and of phosphorus pentoxide were apparently less active (or inactive) from cholestene and more active from cholesterilene than from cholesterol.

SUMMARY

The sterol color reactions were reviewed and the use of various reagents in chemical antirachitic activation and in sterol color reactions was studied. Color reactions, involving addition compound formation, esterification, or oxidative reagents, are probably not involved in chemical activation. Chemical activation by means of acidic dehydrating reagents used in sterol color reactions is correlative with color reactions. Various factors such as temperature, time, solvent, proportion of the reagent, anhydrous condition of the reaction mixture, structure of the compound treated, and character of the reagent used are important in chemical activation and in color reactions.

In general the reaction products obtained from cholesterilene are more active and those from cholestene are probably less active than those produced from cholesterol. Sulfuric acid with acetic anhydride and zinc chloride with acetyl chloride were the most effective reagents in the conversion of cholesterilene to antirachitic products. The color change observed and the activation produced by the treatment of cholesterol with sulfuric acid and acetic anhydride were effected in less time when the reaction mixture was irradiated.

BIBLIOGRAPHY

1. Eck, J. C., and Thomas, B. H., *J. Biol. Chem.*, **119**, 631 (1937).
2. Eck, J. C., and Thomas, B. H., *J. Biol. Chem.*, **119**, 621 (1937).
3. Eck, J. C., Thomas, B. H., and Yoder, L., *J. Biol. Chem.*, **117**, 655 (1937).
4. Eck, J. C., and Thomas, B. H., *J. Biol. Chem.*, **128**, 257 (1939).
5. The pharmacopœia of the United States of America, eleventh decennial revision, Easton, 478–484 (1936).
6. Eck, J. C., and Van Peursem, R. L., *Iowa State Coll. J. Sc.*, **13**, 115 (1939).
7. Salkowski, E., *Arch. ges. Physiol.*, **6**, 207 (1872); *Z. physiol. Chem.*, **57**, 515 (1908).
8. Nikalaev, V. I., and Krastelevska, S. A., *J. Russ. Physic.-Chem. Soc.*, **60**, 1211 (1928).
9. Whitby, G. S., *Biochem, J.*, **17**, 5 (1923).

10. Wokes, F., *Biochem. J.*, **22**, 830 (1928).
11. Drummond, J. C., and Watson, A. F., *Analyst*, **47**, 341 (1922). Fearon, W. R., *Biochem. J.*, **19**, 888 (1925).
12. Hesse, O., *Ann. Chem.*, **211**, 284 (1881).
13. Bills, C. E., and McDonald, F. G., *J. Biol. Chem.*, **72**, 1 (1927).
14. Neuberg, C., *Z. physik. Chem.*, **47**, 335 (1906). Montignie, E., *Bull. soc. chim.*, **47**, 467 (1930). Mandel, J. A., and Neuberg, C., *Biochem. Z.*, **71**, 186 (1915).
15. Liebermann, C., *Ber. chem. Ges.*, **18**, 1863 (1885).
16. Burchard, H., *Chem. Zentr.*, **61**, (I), 25 (1890). Schube, P. G., *J. Lab. and Clin. Med.*, **18**, 306 (1932). Morgareidge, K., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **109**, p. lxvii (1935). Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933). Pijoan, M., and Walter, C. W., *J. Lab. and Clin. Med.*, **22**, 968 (1937). Latarjet, R., and Husson, A., *Compt. rend. Soc. biol.*, **125**, 683 (1937).
17. Lifschütz, I., *Ber. chem. Ges.*, **41**, 252 (1908); *Z. physiol. Chem.*, **117**, 201 (1922).
18. La Lande, W. A., Jr., *J. Am. Chem. Soc.*, **55**, 1536 (1933). Radsma, W., *Acta brev. Neerl. Physiol. Pharmacol., Microbiol.*, **5**, 67 (1935). Levine, V. E., and Bien, G. E., *Proc. Soc. Exp. Biol. and Med.*, **31**, 804 (1934). Levine, V. E., and Richman, E., *Proc. Soc. Exp. Biol. and Med.*, **31**, 582 (1934).
19. Tschugaeff, L., *Z. angew. Chem.*, **13**, 618 (1900).
20. Steinle, J. V., and Kahlenberg, L., *J. Biol. Chem.*, **67**, 425 (1926).
21. Kahlenberg, L., *J. Biol. Chem.*, **52**, 217 (1922).
22. Goldhammer, H., and Kuen, F. M., *Biochem. Z.*, **215**, 6 (1929).
23. Montignie, E., *Bull. soc. chim.*, **45**, 302 (1929).
24. Carr, F. H., and Price, E. A., *Biochem. J.*, **20**, 497 (1926).
25. Moore, T., and Willimott, S. G., *Biochem. J.*, **21**, 585 (1927).
26. Rosenheim, O., *Biochem. J.*, **27**, 386 (1927).
27. Barr, T., Heilbron, I. M., Parry, E. G., and Spring, F. S., *J. Chem. Soc.*, 1437 (1926).
28. Marston, H. R., *Australian J. Expt. Biol. and Med. Sc.*, **1**, 53 (1924).
29. Rosenheim, O., and Drummond, J. C., *Biochem. J.*, **19**, 753 (1925).
30. Rosenheim, O., *Biochem. J.*, **21**, 386 (1927).
31. Rosenheim, O., *Biochem. J.*, **23**, 47 (1929).
32. Schoenheimer, R., and Evans, E. A., Jr., *J. Biol. Chem.*, **114**, 567 (1936).
33. Rosenheim, O., and Callow, R. K., *Biochem. J.*, **25**, 74 (1931).
34. von Zijp, V., *Pharm. Weekblad*, **70**, 775 (1933).
35. Tortelli, M., and Jaffe, E., *Chem. Ztg.*, **39**, 14 (1915). Hanssler, E. P., and Branchli, E., *Helv. chim. acta*, **12**, 187 (1929). Heilbron, I. M., and Spring, F. S., *Biochem. J.*, **24**, 133 (1929).
36. Montignie, E., *Bull. soc. chim.*, **51**, 690 (1932).
37. Bahl, E., *Biochem. Z.*, **204**, 474 (1929).
38. Montignie, E., *Bull. soc. chim.*, **53**, 1394 (1933).
39. Bills, C. E., *J. Biol. Chem.*, **67**, 753 (1926).
40. Obermüller, K., *Z. physiol. Chem.*, **15**, 37 (1891).
41. Bloch, A., *Biochem. Z.*, **267**, 171 (1933).

URINARY EXCRETION PRODUCTS OF SULFANILYL-2-AMINOPYRIDINE*

By HERMAN D. RATISH, JESSE G. M. BULLOWA, JAMES B. AMES, AND JOHN V. SCUDI

(*From the Littauer Pneumonia Research Fund, New York University, and the Medical Service, Harlem Hospital, New York*)

(Received for publication, January 4, 1939)

Although it has been known for some time (1, 6) that sulfanilyl-2-aminopyridine when administered to human beings is excreted partly in the conjugated form of the acetyl derivative, no details of the isolation and characterization of this substance have been reported. The acetyl derivative is readily isolated from urine. Details of a typical run are presented here.

A sample of urine (250 cc.) was obtained from a patient receiving 1 gm. of the drug orally every 4 hours for 3 days. The concentration of the drug in this specimen was 100 mg. per cent free and 51 mg. per cent conjugated sulfanilyl-2-aminopyridine. The blood concentration at the time of micturition was 6 mg. per cent of the free drug. These values were obtained by Marshall's method (2) for the determination of sulfanilamide, although the chromotropic acid method (3) may be used.

The urine was diluted to 1 liter with 95 per cent alcohol, 150 gm. of animal charcoal (S. B. Penick and Company, powdered) were added, and the mixture was shaken for 5 minutes at pH 7. The mixture was centrifuged and filtered. The charcoal was shaken with four successive portions of alcohol (250 cc.), the mixture being centrifuged and filtered after each extraction. A fifth test extraction gave a negative coupling reaction before and

---

* These studies received financial support from the Littauer Pneumonia Research Fund, from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

Samples of sulfanilyl-2-aminopyridine were kindly furnished by Merck and Company.

after hydrolysis. The combined filtrates were evaporated to dryness on a water bath. The residue was cooled and extracted with 10 cc. of cold water. This left an organic residue which gave a negative coupling reaction for the free, but a positive reaction for the conjugated sulfanilyl-2-aminopyridine. After three crystallizations from alcohol 94 mg. of the total 127 mg. of the conjugated drug were obtained. The melting range was 220–222°. Two additional crystallizations from alcohol and a final crystallization from dilute acetic acid gave a product in excellently formed parallelogram-shaped plates, melting at 225–226°. This melting point agrees with that of the acetylsulfanilyl-2-aminopyridine recorded by Marshall (1). The substance was difficultly soluble in cold water, soluble in acids and alkalies, and gave a negative diazo test which became positive after hydrolysis. These are the properties of acetylsulfanilyl-2-aminopyridine. These properties account for the functional groups present in the compound.

$C_{13}H_{13}O_3N_3S$. Calculated. C 53.61, H 4.47, N 14.43
Found. " 53.78, " 4.63, " 14.18

The monoacetylsulfanilyl-2-aminopyridine was prepared in essentially quantitative yields by simply stirring 1 gm. of the drug into solution in 10 cc. of cold acetic anhydride. This procedure is analogous to that reported for the preparation of acetylsulfanilamide (4). After 3 to 5 minutes the product precipitated. The reaction mixture was diluted to a convenient volume with ether and filtered. After crystallization the product was obtained as white parallelogram-shaped plates melting at 225–226°. Mixture of this substance with a portion of the product obtained from the urine specimen did not cause a depression in the melting point.

Warming the drug in acetic anhydride solution at the boiling point for 1 to 20 minutes gave essentially quantitative yields of the diacetyl derivative when the reaction mixture was worked up as in the preceding case. Unlike the diacetyl derivative of sulfanilamide (5) this product melted at a lower temperature than the monoacetylsulfanilyl-2-aminopyridine. After one recrystallization from alcohol the melting point was not altered by two recrystallizations from dilute acetic acid and three from alcohol. The product, appearing in excellently formed long plates, parallelogram in shape, melted at 218°. The melting point was depressed when this product was mixed with a sample of the product obtained from urine, or the synthetic monoacetyl derivative.

$C_{13}H_{13}O_3N_3S$. Calculated, N 12.61; found, N 12.48

A portion of the product isolated from the urine was boiled in acetic anhydride solution. The substance obtained melted at 218° and at the same temperature when mixed with a known sample of diacetyl sulfanilyl-2-aminopyridine.

By the diazotization procedures, the original 10 cc. water extract of the urine residue was found to contain 80 mg. of diazotizable drug. This appears to be considerably above the solubility of the unchanged drug (6). The charcoal adsorbent retained the major portion of the free sulfanilyl-2-aminopyridine, but details of these phases of the study are not ready for publication at this time.

SUMMARY

Monoacetylsulfanilyl-2-aminopyridine has been isolated from human urine after the oral administration of sulfanilyl-2-aminopyridine. This substance has been characterized by physical, chemical, and analytical data and by conversion to the diacetyl derivative. The isolation of other urinary excretion products is under investigation.

BIBLIOGRAPHY

1. Marshall, E. K., Jr., Bratton, A. C., and Litchfield, J. T., Jr., *Science*, **88**, 597 (1938).
2. Marshall, E. K., Jr., *J. Biol. Chem.*, **122**, 263 (1938-39).
3. Scudi, J. V., *J. Biol. Chem.*, **122**, 539 (1938-39).
4. Scudi, J. V., *J. Am. Chem. Soc.*, **59**, 1480 (1937).
5. Scudi, J. V., *Ind. and Eng. Chem.*, *Anal. Ed.*, **10**, 346 (1938).
6. Whitby, L., *Lancet*, **2**, 1095 (1938).

FAT METABOLISM FOLLOWING LIVER INJURY. DECREASED IODINE NUMBER OF TISSUE FATTY ACIDS OF MALE RATS FOLLOWING CARBON TETRACHLORIDE ADMINISTRATION

BY IRWIN C. WINTER

(*From the Department of Physiology and Pharmacology, Baylor University, College of Medicine, Dallas*)

(Received for publication, November 25, 1938)

It has been reported that dogs suffering from liver damage show a decrease in the iodine number of the blood fatty acids during fasting (1, 2). The present series of experiments was designed to determine whether a similar change could be detected in the tissues of animals with liver injury.

Plan of Experiment

Because of their size, albino rats were chosen as the experimental animal. This species has been shown by Cameron and Karunaratne (3) to exhibit liver damage when injected with carbon tetrachloride. To exclude any sex difference (4), only male animals were selected.

Two groups of twelve each were used, six rats of each group being injected with 0.05 cc. of carbon tetrachloride (diluted 1:1 with olive oil to prevent irritation) per 100 gm. of body weight—an amount sufficient in repeated doses to cause severe liver damage—at the start, and on the 2nd, 4th, 7th, 9th, etc., days of the experimental period. The remaining animals served as normal controls. After a preliminary 24 hour fast, each group was placed on a diet containing approximately 10 per cent of fatty acids,[1] the average iodine number of which was 41. All animals were placed in individual wire cages and allowed food and water *ad*

---

[1] Coconut oil added to ground Tioga dog food (Baloration, Inc., Waverly, New York). The composition according to the manufacturer is as follows: protein 20.0, fat 2.5, fiber 3.0, ash 12.5, moisture 8.5 per cent. The ration contains all necessary vitamins, minerals, etc.

libitum, although the amount of food eaten was recorded. The feces were collected and the animals weighed at the time the treated animals were injected with carbon tetrachloride.

At the end of a 2 week feeding period, all of the animals of Group I were sacrificed. Group II was utilized to determine the effect of a fat intake of higher iodine number on the degree of unsaturation of the tissue fatty acids of the animals with liver injury. From this group (II) one normal and one treated rat were killed at the end of the 2 week period, and the remainder placed on a diet[2] which contained fatty acids having an average iodine number of 113. After 2 and 4 days on this diet one treated rat was killed (in each case a normal control was also taken), and the remaining animals were sacrificed after 1 week on the diet with fat of high iodine number. Before being killed or the diet changed, all animals were subjected to a 24 hour fast.

Methods

The feces, liver, and carcass were extracted first with alcohol, then with ether—the feces and liver in continuous extractors, the carcass by refluxing with the solvents named. The feces were ground, moistened with 15 per cent hydrochloric acid, and then dried before extraction.

The resulting alcohol-ether extracts were analyzed for total fatty acids by the method of Bloor (5) and for phospholipid fatty acids by the procedure first used by the same author (6), and the iodine numbers of the fatty acid fractions were determined as described by Yasuda (7). Phospholipid estimations were not attempted on the extracts of the feces.

Results

It is apparent from an examination of Tables I and II and Fig. 1 that on a fat intake of low iodine number liver injury results in a wide-spread interference with the mechanism by which the organism maintains the usual degree of unsaturation of tissue fatty acids. The resultant decrease in iodine number occurs in both total and phospholipid fatty acid fractions, in the liver as well as in the remaining tissues of the body (Table I). The fatty acids appearing in the feces also have a lowered iodine number

---

[2] Cod liver oil added to ground Tioga dog food.

(Table II, Fig. 1), and the constancy of these findings is indicated by the fact that the decreased iodine number of the fecal fatty acids is consistently found in 144 observations on twenty-four animals over a period of 2 weeks. It is concluded that these findings indicate that severe liver injury interferes with the desaturation processes known to occur in the body (8), and that therefore desaturation of fatty acids occurs to a large extent in the liver.

TABLE I

Iodine Numbers of Tissue Fatty Acids

Group No.	Rat No.	Days on Diet 113*	Liver				Carcass			
			Total fatty acids		Phospholipid fatty acids		Total fatty acids		Phospholipid fatty acids	
			Normal	Treated	Normal	Treated	Normal	Treated	Normal	Treated
I	1	0	97	97	109	109	57	54	97	84
	2	0	113	94	137	117	60	45	99	95
	3	0	121	102	129	116	58	50	102	76
	4	0	107	105	131	107	67	51	111	82
	5	0	122	102	145	110	54	38	96	89
	6	0	105	97	132	108	52	38	97	86
Average.....		0	111	100	131	111	58	46	100	85
II	1	0	110	105	128	122	58	45	104	95
	2	2	129	113	149	121		42		95
	3	4	122	126	147	143	63	47	101	93
	4	7	128	125	150	146	62	46	112	99
	5	7	127	126	145	144	55	56	107	90
	6	7	125	129	144	142	55	51	100	91
Average (4, 5, 6).......		7	127	127	146	144	57	51	106	93

* Diet 113 contained fat having a high iodine number. All the rats had been fed Diet 41, which contained fat having a low iodine number, for 14 days before being killed (Group I and Rat 1 of Group II) or placed on Diet 113.

While the number of animals observed on the diet containing fat with a high iodine number is too small to admit of final conclusions, it is definitely indicated that even in severe liver damage the ingestion of highly unsaturated fatty acids can restore to some extent the decreased unsaturation of tissue fatty acids brought about by liver injury. Thus (Table I), total and phospholipid

TABLE II

Iodine Numbers of Fecal Fatty Acids

The fatty acids of the diet had an average iodine number of 41.

	Days on diet	Group I						Group II						Daily average I No.
		Rat No.						Rat No.						
		1	2	3	4	5	6	1	2	3	4	5	6	
Normal rats	2	52	47	52	47	41	51	50	51	55	49	38	41	48
	4	53	48	44	51	44	45	54	59	54	41	36	39	47
	7	49	43	48	50	44	48	50	48	50	44	47	40	47
	9	50	45	49	50	43	48	48	51	53	48	49	43	48
	11	58	46	45	56	49	46	58	42	57	45	49	41	49
	14	47	40	46	45	46	46	58	58	50	55	48	47	49
Average..........		51	45	47	49	44	47	53	52	53	47	45	42	
Treated rats (CCl$_4$)	2	37	25	35	35	36	31	43	36	47	35	45	39	37
	4	43	34	40	45	34	39	39	39	38	35	35	40	39
	7	38	33	46	37	38	35	35	35	40	34	37	33	37
	9	31	28	38	39	30	37	36	39	46	24	37	42	36
	11	39	36	43	40	41	30	41	41	43	37	36	32	38
	14	40	29	46	40	34	40	43	38	46	35	39	43	39
Average..........		38	31	41	39	36	35	40	38	43	33	38	38	

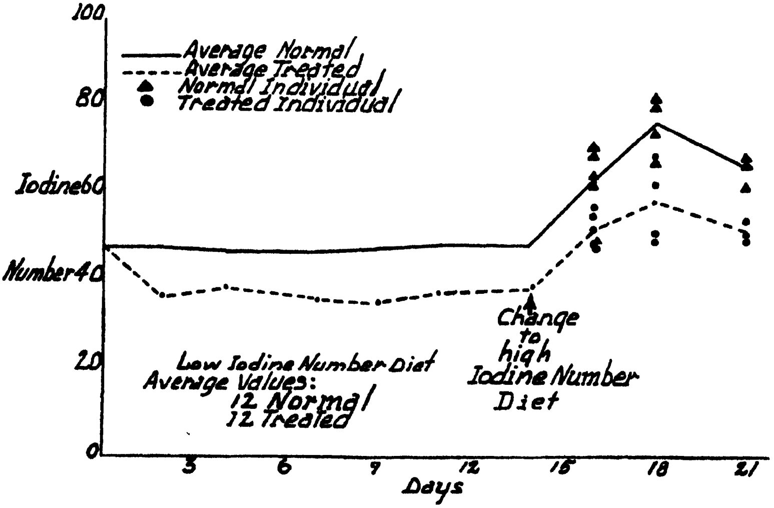


FIG. 1. Change in iodine number of fecal fatty acids of rats on diets of low and high iodine numbers.

fatty acids of the liver are brought to normal iodine values. The rise in iodine number of the fecal fatty acids (Fig. 1) is proportionate to the rise observed in normal animals, but the values do not reach normal levels. (As before, the constancy of the observations concerning the fecal fatty acids (twenty-four observations on ten animals) strongly supports the conclusions given above.) From this last observation it is concluded that the fatty acids found in feces are not from unabsorbed food fat, for in that case the iodine number of these acids from the treated animals should have equaled normal values when the fat with high iodine number was fed. Also, the normal values should have equaled the iodine number of the ingested fatty acids, but were actually higher when the fat with low iodine number was fed, and lower when that with higher iodine number was ingested. Fecal fatty acids must therefore represent *excreted* fat, ingested fat apparently being absorbed 100 per cent even when severe liver damage exists.

SUMMARY

1. Liver injury causes a decreased iodine number of the total and phospholipid fatty acids of the liver and carcass and of the total fatty acids of the feces of male albino rats, when a diet containing a fat of low iodine number is fed over a period of 2 weeks. It is concluded that desaturation processes occurring in the liver are deranged.

2. When such animals (with liver injury) ingest for 1 week a diet containing more unsaturated fatty acids, the iodine numbers of the liver fatty acid fractions rise to normal values.

3. Evidence is presented which supports the theory that fecal fatty acids are largely excreted, rather than unabsorbed food fatty acids.

BIBLIOGRAPHY

1. Winter, I. C., *J. Biol. Chem.*, **124**, 339 (1938).
2. Winter, I. C., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **123**, 218 (1938).
3. Cameron, G. R., and Karunaratne, W. A. E., *J. Path. and Bact.*, **42**, 1 (1936).
4. Deuel, H. J., Jr., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **100**, p. xxxv (1933).
5. Bloor, W. R., *J. Biol. Chem.*, **77**, 53 (1928).
6. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
7. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
8. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **113**, 505 (1936).

DENATURATION AND MOLECULAR SPLITTING OF EGG ALBUMIN BY ULTRAVIOLET RADIANT ENERGY*

By F. W. BERNHART

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis)

(Received for publication, January 12, 1939)

Denaturation of proteins, either in solution or in the dry state, is a well recognized effect of ultraviolet radiant energy (Bovie (1), Stedman and Mendel (2), and Clark (3)). That breakage of the polypeptide chain, with the formation of secondary protein derivatives, also occurs during ultraviolet irradiation is indicated by the results of Becker and Szendrö (4), Spiegel-Adolf (5), and Mitchell (6). Boyd (7) found that the formation of protein split-products during ultraviolet irradiation of fibrinogen and serum albumin occurred only in the presence of oxygen and a photosensitizer. No demonstrable amounts of secondary protein derivatives were found upon irradiation of egg albumin. The present findings disagree with Boyd's results in regard to egg albumin. The data presented in this paper deal with the kinetics of denaturation and molecular splitting of egg albumin, due to ultraviolet irradiation, in solutions exposed to air and under nitrogen.

Methods and Apparatus

A Burdick air-cooled quartz mercury arc was used as a source of ultraviolet radiant energy. This instrument was operated with a voltage drop of 70 volts across the arc. All solutions were irradiated at a distance of 19.5 cm. from the light source. Two ultraviolet quartz mercury arcs varying greatly in efficiency were used during the experimental work. A constant temperature of

---

* The data in this paper are taken from a thesis submitted by the author to the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

25° ± 1° in the irradiated solutions was maintained by the use of a constant temperature water bath.

Egg albumin was crystallized and recrystallized twice by Cole's (8) method. Salts were removed by simple dialysis, followed by electrodialysis, with the apparatus described by Bernhart, Arnow, and Bratton (9). The product, dried over P_2O_5, contained 15.13 per cent nitrogen, as determined by the Kjeldahl method. After heating a solution of the egg albumin for 20 minutes in a boiling water bath, 98.0 per cent of the total nitrogen was found in the isoelectric precipitate. This result agrees closely with the percentage of heat-coagulable protein in crystalline egg albumin solutions, 97.8, found by Bull (10).

Egg albumin solutions (7 to 8 per cent), stored at 5–7° and preserved with a toluene-paraffin oil layer, were removed from the storage flask by a bottom side arm to prevent contamination from the surface layer of preservative. An accurately measured volume (about 10 cc.) was diluted to 100 cc. with distilled water and a definite volume of dilute HCl or NaOH. The pH of this solution was determined by the glass electrode and the egg albumin concentration estimated from nitrogen determined by the Kjeldahl method. Identical fresh solutions were prepared immediately before each irradiation.

18.1 cc. of egg albumin solution were placed in quartz test-tubes (20 × 130 mm.) of approximately 25 cc. capacity. Rubber stoppers fitted with bent glass tubes which allowed free entrance of air were inserted. When the irradiations were carried out under nitrogen, the dissolved oxygen was removed by evacuation and water-saturated nitrogen was bubbled through the solution. 0.02 cc. of *n*-heptyl alcohol was added to prevent surface denaturation of the egg albumin (see Bull and Neurath (11)).

Protein insoluble at the isoelectric point was determined by titrating the irradiated protein solution to pH 4.8 by means of the glass electrode. The contents of the electrode vessel were transferred to a tared, Jena, sintered glass filter crucible (porosity No. 2) and filtered with suction. The precipitated protein was washed twice with 10 cc. portions of water, dried at 105° to constant weight, cooled in a desiccator, and weighed.

EXPERIMENTAL

After irradiation of identical samples of egg albumin solutions for various lengths of time, the protein solutions were titrated to pH 4.8 and the weight of precipitated protein was determined. Three series of protein solutions of different initial pH values were irradiated in contact with air and one series was irradiated under nitrogen.

Examination of the data showed there were no instances in which 100 per cent of the heat-coagulable protein of the solution was insoluble at the isoelectric point. The amount of protein insoluble at the isoelectric point reached a maximum after a few hours of irradiation and then decreased slowly. These results indicate that denaturation of egg albumin by ultraviolet light does not follow a simple first order process, as described by Clark (3). Apparently two opposing processes are occurring at the same time.

If the assumptions are made that (1) denatured protein, insoluble at pH 4.8, is formed by a first order process from undenatured protein present, and (2) protein derivatives, soluble at pH 4.8, are formed at a first order rate from denatured protein present, then

$$dD/dt = k_1S - k_2D \tag{1}$$

where D = denatured protein present at time t
t = time in hours
S = undenatured, unhydrolyzed protein present at time t
k_1 = first order velocity constant of denaturation
k_2 = first order velocity constant of formation of isoelectric-soluble protein derivatives

Now

$$S = S_0e^{-k_1t} \tag{2}$$

where S_0 = total heat-coagulable protein; also undenatured, unhydrolyzed protein at $t = 0$. Then, with Equation 2 substituted in Equation 1,

$$dD/dt = k_1S_0e^{-k_1t} - k_2D \tag{3}$$

On integration Equation 3 yields

$$D = \frac{k_1}{k_2 - k_1} S_0(e^{-k_1t} - e^{-k_2t}) \tag{4}$$

The unknowns in this equation are k_1 and k_2. Data were taken from several points on the denatured protein-time curves and the resulting equations solved for k_1 and k_2. The agreement between calculated and experimental values is shown in Table I.

TABLE I

Weight of Protein Precipitated at pH 4.8 after Irradiation

Description of experiment	Atmosphere	Time irradiated	Observed	Calculated
		hrs.	*mg.*	*mg.*
Ultraviolet Arc 1W, egg albumin concentration 8.1 mg. per cc., initial pH 3.94, $k_1 = 0.08$, $k_2 = 0.0017$, $S_0 = 144$ mg.	Air	6	55	55
		12	92	88
		24	114	119
		36	124	130
		48	130	132
		72	131	130
		96	124	125
		144	114	115
		240	100	98
Ultraviolet Arc 1W, egg albumin concentration 8.1 mg. per cc., initial pH 9.03, $k_1 = 0.053$, $k_2 = 0.0026$, $S_0 = 142$ mg.	"	6	37	39
		12	67	66
		24	102	98
		36	116	114
		48	114	120
		72	115	120
		144	96	102
		240	87	80
Ultraviolet Arc 2S, egg albumin concentration 7.6 mg. per cc., initial pH 6.40, $k_1 = 0.20$, $k_2 = 0.010$, $S_0 = 137$ mg.	"	5	82	83
		10	116	111
		12.5	123	116
		15	118	117
		20	121	116
		25	106	111
		35	95	101
		41	97	96
Ultraviolet Arc 2S, egg albumin concentration 7.6 mg. per cc., initial pH 6.40, $k_1 = 0.18$, $k_2 = 0.0024$, $S_0 = 137$ mg.	N_2	10	109	112
		20	130	128
		40	118	126
		52	123	122
		80	113	114
		100	108	109
		140	111	99
		190	93	88

The calculated values approximate the analytical values fairly satisfactorily. This agreement between experimental and calculated values indicates that the radiant energy processes occurring during ultraviolet irradiation are similar or identical in nature to those assumed to occur. The calculations of Lewis (12) indicate that, at pH values of 3.94, 6.40, and 9.03, the protein denaturation due to acid or base will not interfere in calculations of the protein denatured by ultraviolet light.

Probably the pH change in the irradiated solutions is a complicating factor, causing some change in the first order velocity constants. The best agreement between calculated and experimental values is in the series at initial pH 3.94. The greatest change observed in this series was +0.33 pH. The poorest agreement between the theoretical and experimental data is in the series at pH 9.03, in which the greatest change observed was −3.81 pH. The greatest change of pH observed in the series under nitrogen, initial pH 6.40, was +1.28 and in the parallel series irradiated in contact with air −0.73.

TABLE II

Nitrogen Partition after Irradiation

Initial pH	Time irradiated	Ultraviolet arc No.	Pptd. at pH 4.8	Dialyzable	Not pptd., not dialyzable, experimental
	hrs.		*mg.*	*mg.*	*mg.*
9.03	240	1W	60.2	36.5	4.3
3.43	178	2S	37.7	59.0	
4.24	75	2S	79.3	12.9	10.5

After the denatured protein had been precipitated isoelectrically, the filtrates of the irradiated solutions were investigated. The filtrates, following short periods of irradiation, contained undenatured egg albumin which could be precipitated by heat or sulfosalicylic acid. After longer irradiation no heat-coagulable protein could be demonstrated in the filtrate. Filtrates were investigated after long periods of irradiation to insure the absence of undenatured protein.

The filtrate materials were soluble at pH 4.8, were not heat-coagulable, and did not form a precipitate upon addition of sulfosalicylic acid. The biuret test was positive and the addition of tannic acid resulted in the formation of a voluminous precipitate. A slight precipitate formed when the solution was half saturated with ammonium sulfate. This precipitate was not materially increased upon saturation of the solution with ammonium sulfate. These tests indicate the presence of secondary protein derivatives.

Aliquots of the filtrate and washings were dialyzed under toluene through Visking sausage casing for 36 to 48 hours in a small continuous extraction dialyzer, similar in design to the apparatus described by Bernhart, Arnow, and Bratton (9). H_2SO_4 was placed in the distillation flask to retain nitrogen. The dialyzable nitrogen was determined by nitrogen analyses of aliquots of the distillation flask contents. The non-dialyzable nitrogen was determined by washing the Visking casing with 0.025 N NaOH and analyzing an aliquot of the resulting solution for nitrogen.

The results obtained are shown in Table II.

DISCUSSION

After ultraviolet irradiation of egg albumin it is possible to demonstrate in the filtrate at the isoelectric point the presence of products which can be dialyzed. The precipitation reactions of these products indicate that they are secondary protein derivatives. The most logical assumption concerning their formation is that they are the result of the breakage of the polypeptide chain.

Mirsky and Pauling (13) believe denaturation caused by ultraviolet radiant energy is a process different from denaturation by the action of heat or chemical denaturants. Denaturation by ultraviolet light, according to Mirsky and Pauling, is caused primarily by a photochemical breakage of the polypeptide chain.

If denaturation and hydrolysis are both due to the same cause (breakage of the polypeptide chain), it might be expected that a constant ratio between the first order velocity constant of denaturation and hydrolysis would be observed in these experiments.

The velocity constants of denaturation in samples of the same solution (pH 6.40) irradiated under identical conditions, some of which were irradiated under nitrogen and others in contact with air, were found to be similar (0.20 and 0.18). The velocity constants of formation of protein derivatives in the two series of irradiations were widely different.

In the solutions having access to air this constant was found to be 0.01 and in the solutions covered by nitrogen it was found to be 0.0024. The difference in the ratios between the constants of formation of protein derivatives and the constants of denaturation in the two series due presumably to presence or absence of oxygen indicates that the radiant energy reactions resulting in denaturation and formation of secondary protein derivatives are different in character. The denaturation reaction or reactions are apparently independent of the presence of oxygen, whereas the second reaction or reactions are influenced considerably by the presence of oxygen. The large differences in the ratios of the two constants caused by variations in initial pH value also indicate that denaturation and molecular splitting are due to different processes.

SUMMARY

1. Secondary protein derivatives are formed by the ultraviolet irradiation of egg albumin at 25°.

2. The weights of egg albumin precipitated at the isoelectric point after ultraviolet irradiation follow closely the theoretical values obtained from an equation which assumes that denatured protein is formed by a first order process and that isoelectric-soluble protein derivatives are formed from the denatured protein by a first order process.

3. The presence of oxygen has little influence on the velocity constant of denaturation caused by ultraviolet radiant energy. The velocity constant of the formation of protein derivatives which are soluble at the isoelectric point is increased by the presence of oxygen.

The author wishes to thank Dr. L. Earle Arnow for his assistance in the interpretation of the data presented in this paper.

BIBLIOGRAPHY

1. Bovie, W. T., *Science*, **37**, 373 (1913).
2. Stedman, H. L., and Mendel, L. B., *Am. J. Physiol.*, **77**, 199 (1926).
3. Clark, J. H., *J. Gen. Physiol.*, **19**, 199 (1935).
4. Becker, J. P., and Szendrö, P., *Arch. ges. Physiol.*, **288**, 755 (1931).
5. Spiegel-Adolf, M., *Biochem. J.*, **28**, 372 (1934).
6. Mitchell, J. S., *Proc. Roy. Soc. London, Series A*, **155**, 696 (1936).
7. Boyd, M. J., *J. Biol. Chem.*, **103**, 249 (1933).
8. Cole, A. G., *Proc. Soc. Exp. Biol. and Med.*, **30**, 1162 (1933).
9. Bernhart, F. W., Arnow, L. E., and Bratton, A. C., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 387 (1937).
10. Bull, H. B., *J. Biol. Chem.*, **125**, 585 (1938).
11. Bull, H. B., and Neurath, H., *J. Biol. Chem.*, **118**, 163 (1937).
12. Lewis, W. C. M., *Chem. Rev.*, **8**, 94 (1931).
13. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, **22**, 439 (1936).

THE DEPOSITION OF POTASSIUM AND PHOSPHATE WITH GLYCOGEN IN RAT LIVERS

By W. O. FENN

(*From the Department of Physiology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*)

(Received for publication, January 17, 1939)

In an attempt to investigate the rôle of the liver in absorbing injected potassium it became important to know the effect of variations in glycogen content on the potassium concentration in the liver. Concerning the glycogen, there is clear evidence that it is deposited with such an amount of water that the percentage of water in the whole liver does not change appreciably. This at least is the case in rabbits (MacKay and Bergman, 1932; Bridge and Bridges, 1931, 1932) and in rats (Puckett and Wiley, 1932). In dogs, strangely enough, there seems to be equally good evidence that glycogen, like fat, is deposited in the liver without water. The water content of the dog liver, according to the data of Kaplan and Chaikoff (1936), is accurately determined by the amount of protein present. There is, therefore, some difference of opinion with regard to the relation between glycogen and water storage and there seems to be no evidence to show how potassium would vary with varying amounts of glycogen.

In this paper are reported the results of some experiments which were designed to show whether glycogen is deposited with water in the liver of rats and whether this additional water is accompanied by the same amount of potassium as is usually found in intracellular liver water. Three groups of rats were used, (1) normal, (2) fasted, (3) fasted and then fed large amounts of carbohydrate. The results show clearly that under the conditions of these experiments the glycogen is deposited with both water and potassium.

Methods

Normal adult rats were used from the stock colony. Some were fasted for 48 hours. After a similar period of fasting others were fed for 20 hours with corn-starch and sucrose *ad libitum.* Water was available at all times. A salt solution was also available during this feeding period which contained the salts of Ringer's solution in 10 times the usual concentration. This was supplied on the theory that it might be impossible to deposit potassium with glycogen unless a source of K was made available in the diet. Water without salt was also available at all times. Males and females were used indiscriminately in about equal numbers.

The rats were killed by stunning and the throat was quickly cut over a funnel so that the blood could be collected in a centrifuge tube with heparin. The blood was centrifuged and the plasma was analyzed for chloride and occasionally for K. After the animal was bled to death the liver was removed. Pieces were cut off and blotted rather firmly to remove blood. As quickly as possible the first sample was dropped into a weighed tube containing hot KOH for glycogen. Two separate samples were weighed on a torsion balance and put into platinum crucibles to be ashed for K analyses. After ashing, aliquots from each sample were analyzed in duplicate. Sometimes a similar sample was taken for Na analysis. One or two samples were taken for chloride analysis and one sample in a weighing bottle for water, fat, and protein nitrogen. This sample was first dried to constant weight in an oven at 100°, then extracted as described by Hastings and Eichelberger (1937) with dry ethyl ether and petroleum ether, the loss of weight being taken as an approximate measure of neutral fat.[1] After fat extraction the remainder was analyzed for total nitrogen by the macro-Kjeldahl method and the protein content was calculated by the use of the factor 6.25 as determined by Addis *et al.* (1936). The nitrogen content of this sample was regularly a little less than the total nitrogen content of the fresh liver, which was also determined in most instances. This difference presumably represented most of the non-protein nitrogen.

---

[1] On account of the large size of the dried samples used for this fat extraction it became evident that the results obtained with this method were too low. For this reason they are not reported.

In the early experiments an estimate was also made of blood remaining in the liver. This was done by cutting a small weighed sample of the liver as finely as possible in a known volume of isotonic NaCl solution. After this suspension had settled for a few seconds, a sample of the supernatant was taken and the number of blood corpuscles was counted with a hemocytometer. From the red cell count of rat blood and the volume of the solution, the blood in the liver could be calculated. Values varying from 0.7 to 3 per cent of the liver were obtained. If the animal was not bled to death before sampling, the results were 3.7 and 5.2 per cent. No correction was made for this blood, the plasma being included in the extracellular water and the cells in the cell water automatically. The small error due to chloride in the cells is of no significance.

Chlorides were determined by the method of Van Slyke as modified by Manery, Danielson, and Hastings (1938) and potassium by the method of Shohl and Bennett with the minor modifications described by Fenn *et al.* (1938). Sodium was estimated by the gravimetric method of Butler and Tuthill (1931), rather large amounts of tissue being required. For glycogen the method of Good, Kramer, and Somogyi (1933) was used, the sugar being titrated by the method of Shaffer and Somogyi (1933). One aliquot was subjected to yeast fermentation and only the fermentable fraction was included as glycogen. Phosphorus was determined by the methods of Fiske and Subbarow (1929), the inorganic P being separated by precipitation with $CaCl_2$. For total P a separate sample was completely ashed in H_2SO_4 and H_2O_2.

Results

The analytical results are given in Table I. Figures from six normal, four fasted, and eight sugar-fed rats are included. Water contents were not significantly different in any of the groups. Neither water nor potassium per gm. of wet weight appeared significantly changed by either fasting or feeding, although the glycogen varied from 0.1 to 14.6 per cent. This is shown by the graphs of Fig. 1 where water contents and potassium contents are plotted against the amount of glycogen. The deposition of glycogen as dry inert matter without other change in the liver would

TABLE I

Analyses of Rat Livers, per 100 Gm. of Wet Weight

Rat No.	Weight of rat	Weight of liver	H_2O	Glycogen	Protein	Potassium (duplicates)		Cl	Plasma Cl	Plasma K	Chloride space	Cell H_2O
						Normal						
	gm.	*per cent body weight*	*gm.*	*gm.*	*gm.*	*m.eq.*	*m.eq.*	*m.eq.*	*m.eq. per cent*	*m.eq. per cent*	*gm.*	*gm.*
1	293	3.0	70.2	3.1	20.8*	9.74	9.74	2.84	10.6	0.60	25.7	44.5
2	228	3.5	68.9	2.6	23.4	9.47	9.64	2.81	10.87		24.8	44.1
3	180	3.6	69.3	4.0	21.1	10.19	9.92	2.71	10.34		25.1	44.2
4	190	3.7	69.1	4.8	20.4	9.72	9.77	2.46	10.18		23.2	45.9
5	233	3.8	69.1	3.6	22.4	9.72	9.70	2.30	10.22	0.67	21.6	47.5
6	192	3.8	69.2	4.6	21.7	9.41	9.43	2.23	9.85		21.7	47.5
	219	**3.6**	**69.3**	**3.8**	**21.7**	**9.70**		**2.56**	**10.34**		**23.7**	**45.6**
						Fasted						
7	162	2.4	68.4	0.2	23.0	10.33	9.85	2.78	10.07	0.67	26.5	41.9
8	138	3.0	70.8	0.5	22.7	9.52	9.92	2.84	10.18		26.8	44.0
9	185	2.4	70.9	0.1	23.8*	10.38	10.20	3.10	10.03		29.6	41.3
10	167	2.6	71.3	0.1	22.1	10.45	10.54	3.32	10.38		30.0	41.3
	163	**2.6**	**70.4**	**0.2**	**22.9**	**10.13**		**3.01**	**10.16**		**28.2**	**42.1**
						Sugar-fed						
11	161	4.3	68.4	8.2	17.7	9.74	9.62	2.54	9.78	0.65	26.8	41.6
12	213	4.0	67.9	12.2	14.0	9.31	9.24	2.98	10.1	0.65	28.4	39.5
13	165	3.5+	69.4	13.3	13.8*	9.39	9.34	2.84	9.79		27.9	41.5
14	176	3.7	69.6	11.6	13.3	9.92	10.1	2.54	9.92		24.7	44.9
15	181	4.5	68.8	11.1	14.4	9.89	9.28	2.46	10.72		22.0	46.8
16	190	4.2	69.7	11.6		9.14	9.80	2.58	10.11		22.9	46.8
17†	165	6.4	69.7	14.1	12.1	9.62	9.26	2.58			24.7‡	45.0
18†	165	5.9	68.8	14.6	13.4	9.55	9.10	2.02	10.04		19.3	49.5
	177	**4.6**	**69.0**	**12.1**	**14.1**	**9.52**		**2.57**	**10.01**		**24.6**	**44.5**

The figures in bold-faced type represent averages.

* Total N × 6.25.

† Fed with casein and sugar.

‡ Calculated from average plasma chloride.

result in a decrease in the concentrations of water and K according to the straight lines marked "dry." If, however, glycogen is deposited together with the same amounts of water and K as are normally associated with other liver solids, then there should be no change in the concentration of either water or K as the glycogen increases. There seems to be a slight decrease in both H_2O and K as the glycogen increases from the fasting to the normal level, but this change is probably not significant. Figures in addition to those in Table I were obtained from two other rats, one normal and one fasted, the latter giving lower values for both K and H_2O. In these animals the K (per 100 gm., dry weight) was 1.18 in the fasted and 1.22 in the normal rat, while the H_2O (per cent wet weight) was 74 in the normal and 72.7 in the fasted animal.

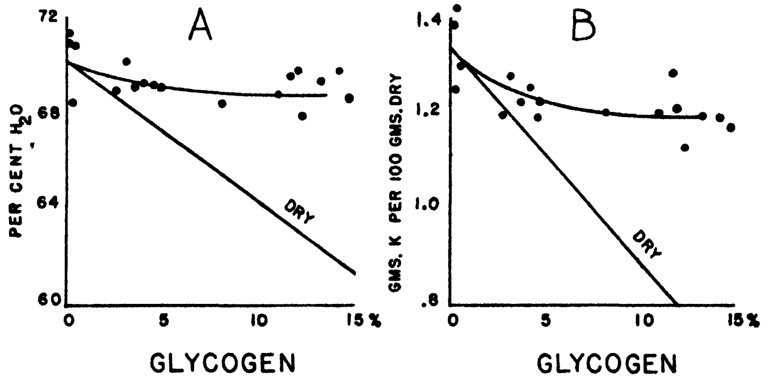


FIG. 1. The deviation of the experimental points from the straight line ("dry") represents (*A*) the amount of water deposited with the glycogen, (*B*) the amount of potassium deposited with the glycogen.

It is frequently observed that liver fat increases in fasting and this might diminish or mask an increase in both H_2O and K. Unfortunately no satisfactory figures for total liver lipids are available for the animals of Table I, but in four other animals similarly treated, two normal livers showed 5.47 per cent, and two livers from fasted rats showed 5.49 per cent total lipids. These are averages of duplicate analyses for each of which a hot alcohol extract plus an ether extract of the fresh ground-up livers was dried and reextracted with petroleum ether. Best and Ridout (1938) state that "stock male rats, weighing approximately 200 gm. usually do not exhibit an increase in liver fat during a 48 hour fast." It is impossible to believe, therefore, that the liver fat could have varied sufficiently to modify significantly the interpretation of the data. The essential constancy of both K and H_2O even at high glycogen levels indicates, therefore, that glycogen is deposited along with both K and H_2O in appropriate amounts.

A similar conclusion is reached if the protein percentage is plotted against the glycogen, as in Fig. 2. If the glycogen is simply inert matter added to the liver, then the protein percentage should decrease according to the upper straight line. If, however, 1 gm. of glycogen is deposited along with 2.33 cc.[2] of water, then the protein percentage should decrease according to the lower straight line. Actually, the experimental points fall closely about the latter line, thus confirming the essential correctness of

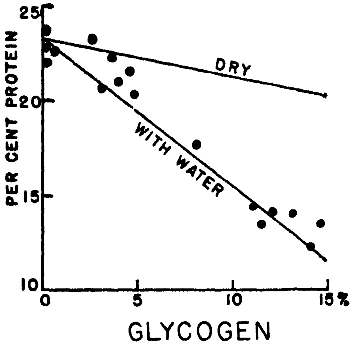


FIG. 2

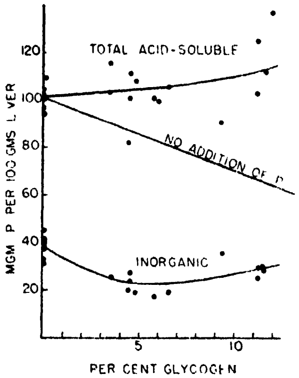


FIG. 3

FIG. 2. The straight lines are drawn on the assumption that glycogen is deposited "with water" or "dry," the protein remaining unchanged in absolute amount. The experimental points show that protein is simply diluted by the deposition of glycogen plus water.

FIG. 3. Phosphorus fractions as affected by glycogen deposition. The straight line marked "no addition of P" represents the concentration of total acid-soluble P expected if the amount present in the absence of glycogen were simply diluted by the deposition of glycogen plus water. The deviation of the experimental points from this line represents the amount of P which is deposited with the glycogen.

---

[2] In a liver containing 70 per cent water each gm. of dry liver has 2.33 cc. of water associated with it. Puckett and Wiley (1932) have concluded that 1 gm. of glycogen is stored with 2.4 cc. of H_2O.

this interpretation. A very similar plot may be obtained from the data of Bridge and Bridges (1931) for rabbits, showing that in this animal as well as in the rat the deposition of glycogen involves an increment in the amount of liver tissue which is much in excess of the actual weight of glycogen added.

The data so far presented might conceivably be explained if glycogen were deposited in exchange for an equal weight of protein. This interpretation, however, is essentially improbable and it will not explain the considerable increase in the size of the liver in per cent of the body weight shown for the carbohydrate-fed rats in Table I where the liver increases from 2.6 to 4.6 per cent of the body weight are reported. Assuming a 160 gm. fasting rat with a liver of the average size and composition indicated in Table I, it may be calculated that an increase in glycogen from 0.2 to 12.1 per cent (average for fed rats) by the deposition of glycogen plus water (2.33 cc. per gm. of glycogen) would increase the size of the liver from 2.6 to 4.3 per cent of the body weight. This figure is not significantly different from the average liver weight of 4.6 per cent actually observed (4.1 per cent if the last two rats which also received some casein are omitted). To attain the same glycogen content by deposition of glycogen without water the liver would have increased only to 2.95 per cent of the body weight.

In the case of Rats 17 and 18, Table I, some casein was mixed with the carbohydrate of the diet. This resulted in a still greater increase in liver weight up to 5.9 and 6.4 per cent of the body weight, possibly because more food was eaten.

From the chloride determinations in liver and plasma, the chloride spaces have been calculated for the three groups of animals.[3] The results in Table I show perhaps a slight increase from 24 up to 28 per cent of the liver weight due to fasting but the value in the livers of fed animals is normal (24.6 per cent). From these figures it is evident that as the liver increases in size from 2.6 to 4.6 per cent of the body weight by enlargement of the cells, the extracellular spaces increase in the same proportion.[4] Of the 2.33 cc. of water deposited with each gm. of glycogen, approximately 0.8 cc. is not actually with the glycogen but is extracellular. If all of the water had been extracellular, the chloride spaces in the livers of fed rats would have been about 45 per cent instead of 25 per cent. If on the other hand, there had been no increase at all in the extracellular phase during the growth of the liver, the chloride space would have been only 16.9 per cent in the livers of fed animals. The significantly larger chloride space actually found cannot be accounted for by increase in blood supply because of the small amounts of blood found in the exsanguinated samples.

---

[3] Chloride space = 100 × 0.96 (liver chloride)/(plasma chloride), where 0.96 is the expected ratio of tissue space Cl to plasma Cl on the basis of a Donnan equilibrium.

Since the percentage chloride space does not change significantly with increase in glycogen, it follows that the cell water remains similarly constant, there being only a slight decrease in the case of fasted animals (last column of Table I). The potassium being also slightly higher in these animals, it is evident that the concentration of K per liter of cell water is slightly higher in fasting. This would be expected, since a larger fraction of the potassium in these livers is combined with protein, which is relatively inactive osmotically, the deficit being made up by more cations.

Since the chloride space remains constant during glycogen deposition and growth of the liver and since the cells grow by intake of water and potassium but without change in protein, it is evident that some other anion must have been taken in to combine with the K. This anion is presumably phosphate, as is shown by results on a second series of rats which were fasted and fed in the same way as those reported in Table I, their livers being analyzed, however, only for glycogen, inorganic P, total acid-soluble P, and total P. The data were obtained from four normal, four fasted, and three sugar-fed rats. Duplicate determinations were made in every case. No large changes in either inorganic or total acid-soluble fractions were found, as is shown by the graphs of Fig. 3. The amount of total acid-soluble P which was added during the deposition of glycogen is indicated by the deviation of the experimental points from the straight line which marks the position of the graph which would have been expected had the original acid-soluble P been merely diluted by the addition of glycogen and water. Average figures from these same animals are shown in Table II, where values for total P are also given. There is a definite decrease in total P as glycogen increases. Since the total acid-soluble P remains practically constant, the acid-insoluble P decreases even more than the total P. The acid-insoluble P is evidently diluted by the glycogen and the water just as the protein is. This is shown clearly in Table II, where the values of acid-insoluble P are calculated on the assumption that the amount present in the livers of fasted animals remains unchanged during the deposition of glycogen and water.

---

[4] Evidence that chloride really represents extracellular water will be published shortly from this laboratory by Truax.

TABLE II

Average Phosphorus Fraction and Glycogen in Rat Livers, per 100 Gm. of Fresh Liver

Condition of animal	Weight of liver	Glycogen	P per 100 gm. fresh liver				
			Inorganic	Total acid-soluble	Total	Acid-insoluble	
						Observed	Calculated
	per cent body weight	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Fasted	2.8	0.04	39	102	417	315	315
Normal	3.2	4.7	22	104	373	269	260
Fed	4.5	9.6	29	111	299	188	214

The differences between the calculated and observed values are not significant.

Sodium was determined in a few cases and the ratio of Na:Cl was found to be 0.92 and 0.88 in two normals, 0.89 in a fasted rat, and 0.73 in one on a high glycogen diet. This indicates less Na than Cl in the chloride spaces or the presence of Cl in some cells where K rather than Na is the chief cation.

It is now well known that when the glucose concentration decreases in the blood after insulin, the K and phosphate concentrations decrease likewise. Assuming a valence of 2 for phosphate, the amounts of these two substances which disappear from the blood are usually approximately equivalent. (Recalculation of data of Harrop and Benedict (1924), of Briggs, Koechig, Doisy, and Weber (1923–24), and of Kerr (1928).) The number of equivalents of sugar disappearing in the same time is frequently not far from the same order of magnitude. This suggests the formation of a potassium hexosephosphate, although it is based only upon net changes in the blood. In the rat livers, however, the amount of glycogen deposited is far greater (in equivalents) than the amount of K deposited along with it, so that only a very small fraction, if any, can be in the form of K hexosephosphate. These experiments afford, nevertheless, a possible explanation for this observed connection between potassium and carbohydrate metabolism.

It must be concluded from these experiments either that rats and dogs differ in their method of deposition of glycogen or that under some conditions glycogen can be deposited without water. It seems possible that the glycogen might become more dry and better condensed after remaining in the liver for longer periods of time. This might explain the results of Kaplan and Chaikoff (1936) who produced changes in liver composition chiefly by the slower process of hormone administration.

SUMMARY

Rat livers were analyzed for H_2O, N, glycogen, fat, Cl, Na, K, inorganic P, acid-soluble P, and total P, and the blood plasma for Cl and K. Glycogen was varied from 0 to 14 per cent by fasting and carbohydrate feeding. Analysis of the results shows that glycogen is deposited with the same amount of H_2O, K, and acid-soluble P as is found associated with the other solids of the liver. The extracellular or connective tissue phase (chloride space) increases in proportion to the increase in the size of the cells. The liver increases in size more than can be explained by the addition of glycogen only. Protein and acid-insoluble P remain unchanged in absolute amounts but their concentrations are diminished by dilution with glycogen and water.

I am indebted to Doris Cobb Marsh and Miss Eugenia Sheridan for much of the analytical work reported in this paper and to Miss Lorraine Haege for assistance with the phosphorus analyses.

BIBLIOGRAPHY

Addis, T., Poo, L. J., Lew, W., and Yuen, D. W., *J. Biol. Chem.*, **113**, 497 (1936).

Best, C. H., and Ridout, J. H., *J. Physiol.*, **94**, 47 (1938).

Bridge, E. M., and Bridges, E. M., *J. Biol. Chem.*, **93**, 181 (1931); **96**, 381 (1932).

Briggs, A. P., Koechig, I., Doisy, E. A., and Weber, C. J., *J. Biol. Chem.*, **58**, 721 (1923–24).

Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, **93**, 171 (1931).

Fenn, W. O., Cobb, D. M., Manery, J. F., and Bloor, W. R., *Am. J. Physiol.*, **121**, 595 (1938).

Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).

Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).

Harrop, G. A., Jr., and Benedict, E. M., *J. Biol. Chem.*, **59**, 683 (1924).

Hastings, A. B., and Eichelberger, L., *J. Biol. Chem.*, **117**, 73 (1937).

Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **116**, 663 (1936).

Kerr, S. E., *J. Biol. Chem.*, **78**, 35 (1928).

MacKay, E. M., and Bergman, H. C., *J. Biol. Chem.*, **96**, 373 (1932); **105**, 59 (1934).

Manery, J. F., Danielson, I. S., and Hastings, A. B., *J. Biol. Chem.*, **124**, 359 (1938).

Puckett, H. L., and Wiley, F. H., *J. Biol. Chem.*, **96**, 367 (1932).

Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).

THE DISTRIBUTION OF POTASSIUM IN THE CAT AFTER INTRAVASCULAR INJECTION

By W. S. WILDE

(*From the Department of Physiology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*)

(Received for publication, February 6, 1939)

The idea that tissue cells, like red blood cells, are impermeable to potassium has now been generally discarded in favor of the theory that potassium can penetrate freely into all tissue cells. For the present it seems safer to take a middle ground and to assume that while some of the tissue cells are certainly permeable to potassium there may be others which are impermeable. The experiments to be reported in this paper were undertaken to throw light upon this question.

The argument upon which the experiments are based is as follows: If A mg. of potassium are injected into an animal of weight W kilos and the increase in the concentration of K in the plasma is x mg. per 100 cc. of plasma, then the volume of distribution of the potassium V_K is taken as $10\ A/xW = V_K$. If $V_K > 30$, then some of the potassium must have penetrated into some of the cells. If $V_K > 70$, then the injected K must have accumulated in some cells in greater amount than in an equal volume of plasma. If $V_K = 70$, this might be taken to mean that the increase in concentration of K in the cell water is equal to x. It might equally well mean, however, that it is $< x$ in some cells and $> x$ in other cells. The latter would seem to be the more conservative conclusion.

Since this work was begun, Winkler and Smith (1938) have reported similar experiments in which it appeared that $V_K = 70$. Bourdillon (1937) reached a similar conclusion by experiments in which K was ingested. The experiments to be reported in this paper confirm this experimental finding and show also that values of V_K in excess of 70 may possibly be due to experimental error.

Experimental proof of the accumulation of injected potassium in certain cells is therefore lacking, although this probably occurs. In addition to yielding a valuable confirmation of previous reports these experiments furnish information concerning the time relations of the disappearance of potassium from the blood after intravascular injection and suggest in addition that some of the potassium which disappears from the blood in 15 to 25 minutes after injection reappears again at 40 minutes before finally being absorbed. The significance of this rise is not apparent.

Method

Cats were usually anesthetized with dial (0.7 cc. per kilo injected intraperitoneally), but a few were decerebrated under ether. The potassium solutions described in Table I were injected during 2 minutes into the common carotid artery or the popliteal vein. Blood samples (1.2 cc.) were taken from the carotid artery and mixed in the syringe with 0.5 mg. of dry heparin measured accurately in a special spoon. The heparin itself contained 1.57 mg. of potassium per gm., an amount insufficient to raise the plasma potassium concentration more than 0.2 mg. per cent. Food was removed from the animal cages 18 hours before an experiment and the potassium was injected 90 minutes after the beginning of anesthesia.

Because of the effect of hemorrhage on blood potassium (Thaler, 1935) it was necessary to take very small samples (0.45 cc. of plasma containing about 0.06 mg. of K). The potassium analysis of Shohl and Bennett as modified by Fenn and Cobb (1935) was further adapted by reducing the precipitating agents to 1 drop of N HCl and 0.06 cc. of 10 per cent platinic chloride. These were mixed with the dry ash in the centrifuge tube and precipitated in 1 cc. of absolute alcohol saturated with K_2PtCl_6. The precipitate was washed twice on the centrifuge with 0.6 cc. of the same alcohol. A thin glass rod identified with the sample was used for mixing. The washed precipitate was incubated with 0.4 cc. of water and 0.5 cc. of 2 N KI and was titrated hot with 0.0025 N $Na_2S_2O_3$ in a microburette graduated to 0.01 cc. Samples containing 0.45 cc. of plasma from a common pool were ashed and analyzed. The average deviation from the mean was 1.7 per cent and the probable error was ±1.4 per cent of the mean. All the blood samples were centrifuged for 15 minutes in tubes of 4 mm. inside diameter and 10 cm. length. The small changes in the cell volumes noted could not be correlated with the potassium injections, which amounted to only about 4 cc. per kilo of cat.

Results

The experimental results are shown in Table I. In most experiments the ureters were ligated and KCl was injected into the carotid artery. Variations from this procedure are indicated in Table I. The first three lines refer to control experiments without added K. Values of plasma potassium concentration are given before injection and at various times from 2 to 90 minutes after injection. The first three columns show the number of the animal, its weight, and the amount of K injected. From these figures the values of the last column have been calculated, these being the values of plasma K which would be expected if the injected potassium had diffused into all the body water or 70 per cent of the body weight. If observed values are at any time less than this, it would indicate an increase in K concentration in some cells greater than the increase observed in the plasma. While many isolated cases of this sort are observed in Table I, it seems possible to explain all of them (except perhaps in Cat 1) either as analytical error or as random fluctuation in the basal level of potassium in each experiment.

In order to treat the results statistically, the initial potassium concentration in each experiment was subtracted from each of the subsequent concentrations and these differences were averaged together for different times after injection. This procedure was considered legitimate, because the weights of the cats and the amounts injected were nearly equal in all the experiments and the differences in procedure appeared to have had no effect upon the results. To obtain enough figures for valid statistical treatment of the averages those of the columns for 5 to 7 minutes and for 10 to 12 minutes were combined, as were also the averages of the 15, 20, and 25 minute columns, the 35 and 45, and the 60 and 90 minute columns. These average figures together with the probable errors of the averages are given in the last line of Table I, and are plotted against time in Fig. 1.

The middle curve in Fig. 1 represents the fall of potassium con-

TABLE I

Plasma Potassium Values Following Injection into Cats

Cat No.	Weight	K injected per kilo	K, mg. per 100 cc. plasma											
			Min. after injection											Calculated for $V_K = 70$
			Before	2-3	5-7	10-12	15	20	25	35	45	60	90	
Dial; ureters ligated; control sampling; no injection														
	kg.	mg.												
11 ♂	3.70		15.6	15.9	15.0		15.0	15.1	16.0	15.4	16.3	16.2	15.2	
15 ♂	2.80		15.5	15.6		14.7	15.4		14.3		14.5	16.0	15.9	
10 ♂	4.46		18.4	18.6		18.4	18.6		18.5	19.1		18.2	18.4	
Dial; ureters ligated; 0.1 N KCl, intracarotid														
5 ♂	2.35	14.6	16.8	22.4	28.4	22.0		21.1		21.5	23.8	22.8	21.1	18.9
6 ♀	2.75	14.2	15.3	18.9	17.9	17.6			17.2	19.2	18.9	19.5		17.3
8 ♀	2.08	15.0	20.0		22.4	22.4	22.4	21.3		22.0	22.8	21.9	23.6	22.1
Dial; ureters ligated; 0.15 N KSCN, intracarotid														
9 ♂	4.03	16.0	15.4	22.5	17.7	18.5	18.1		20.3	20.3		23.0	21.7	17.7
10 ♂	4.45	15.9	18.4	21.4			18.5		22.2			23.1		20.7
14 ♀	2.53	15.8	16.0	25.8		19.8	19.7		19.5	19.5	19.5	19.9	18.9	18.2
Decerebrate; ureters ligated; 0.1 N KCl, intracarotid														
1 ♀	2.70	14.5	16.2	23.2		15.7			14.3			15.4		18.4
2 ♂	2.20	14.2	14.7	19.1	16.2		17.0			15.2		16.5	14.4	16.7
7 ♀	3.50	16.7	15.6			22.2		18.6		19.7		20.0	19.9	18.0

Dial; ureters ligated; 0.15 N KSCN, intravenous														
16 ♀	2.85	20.6	15.3	23.3		19.4		19.3	20.3	20.5	19.7	20.5	19.9	18.2
17 ♀	2.80	16.7	18.4	28.1		23.9	20.9		20.1	24.2	23.2	20.1	19.7	20.8
19 ♀	3.30	14.2	15.4	18.4		16.1	18.1		15.7	15.5	16.0	16.3	15.7	17.4
Dial; artificial respiration; ureters ligated; 0.15 N KSCN, intracarotid														
15 ♂	2.80	15.7	17.8	22.5			19.0			19.9		20.4		20.0
Dial; renal blood vessels ligated; 0.15 N KSCN intracarotid														
13 ♀	2.36	16.1	16.5	21.9		18.5	17.4		17.5	18.3	19.0	19.4	19.7	18.8
11 ♂	3.70	16.2	17.3	22.2			20.2		19.9	19.4		18.5		19.6
Dial; kidney intact; 0.1 N KCl, intracarotid														
3 ♀	3.00	14.4	16.3	24.2	20.8	20.9		18.3		18.8			16.0	
18 ♀	2.15	16.4	19.3	23.3		20.6			21.2	22.3	22.9	18.9	23.0	20.1
3 ♀	3.00	14.4	16.0		17.0	14.6		17.3		17.9		13.7	16.4	16.9
Average ΔK ± P.E. of mean.....				5.9 ± 0.4	3.5 ± 0.4		2.3 ± 0.2			3.3 ± 0.2		2.9 ± 0.28		

Cats 10, 11 and 15 were used first for control sampling and were later injected with potassium and further samples were taken. Two successive injections were made into Cat 3; the results of the second injection were omitted from the average. It is impossible to calculate the potassium level corresponding to $V_K = 70$ when the kidneys were intact, except when the loss in the urine was separately determined and then (last two lines) the value applies only to the 90 minute value. The last line gives the average ΔK in the plasma and the probable error of this mean as described in the text and plotted in Fig. 1.

centration in the blood toward the basal level, the length of the vertical line at each point representing the probable error of the mean. The curve is seen to reach a minimum in 15 to 25 minutes, then a maximum at the 35 to 45 minute point, with a second smaller fall at 60 to 90 minutes. The magnitude of this rise at 40 minutes is 1.0 ± 0.28 mg. per cent where 0.28 is the probable error of the difference between the minimum at 20 minutes and the maximum at 40 minutes ($0.28^2 = 0.2^2 + 0.2^2$). This difference being 3.6 times the probable error, it may be concluded that there is only about one chance in 62 that this secondary rise is accidental and not representative of a real phenomenon.

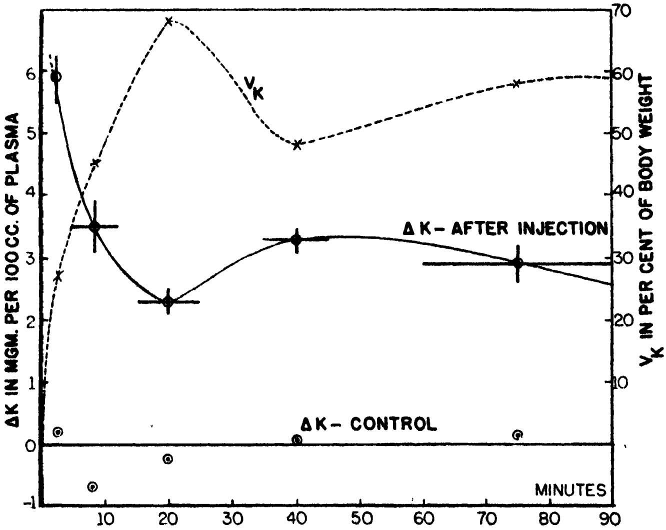


FIG. 1. The average increase in concentration of K in mg. per 100 cc. of plasma at various times after injection. The straight lines drawn through each point represent, vertically, the probable error of the mean and horizontally the times for which averages were taken. Similar averages from three control experiments are shown in the lower graph. The dotted line shows volumes of distribution of K calculated from the corresponding average ΔK.

In Fig. 1 are also plotted the calculated volumes of distribution (V_K) of the potassium corresponding to the average plasma concentrations observed, as well as the variations in plasma concentration observed in the three control experiments (lower graph).

None of the latter control differences is significant. The average volumes of distribution are all under 70 per cent, that at the minimum being 68, that at the maximum being 48 per cent. Isolated points in individual experiments of course give much higher values but cannot be proved significant.

In nine experiments, as indicated in Table I, potassium was injected as KSCN and determinations for SCN were made in the plasma by the method of Lavietes *et al.* (1936). These showed a volume of distribution (V_{SCN}) of 34 per cent as compared to nearly 70 per cent for K. In one experiment the time courses of the diffusion of both K and SCN were followed and it was found that K reached a minimum in 14 minutes, while the SCN continued to decrease for 34 minutes. Potassium evidently penetrates into the potassium (cell) space of the body at least as quickly as SCN penetrates into the chloride space.

DISCUSSION

The important feature of these experiments is the speed with which injected potassium disappears from the circulation. Houssay and Marenzi (1937) have observed a similarly rapid disappearance from the blood of dogs. The absolute amount of potassium which disappears cannot be calculated from their experiments but in cats this calculation indicates that the potassium must be diffusing into some cells against the concentration gradient. Since it has to go against the concentration gradient, it seems unlikely that the increase in concentration in the cells (ΔK_i) will be the same as in the plasma (ΔK_0).

In the experiments of Fenn and Cobb (1934) frog muscles were analyzed for K after immersion in solutions of different K contents. At pH 7.2 (*cf.* their Fig. 1) it was found that the increase in concentration of K in the muscle was 4.0 times the increase in the solution. Allowing for K in an extracellular space of 30 per cent and assuming that 50 per cent of the muscle was cell water, we may calculate that a unit increase in concentration in the solution will cause a 7-fold increase in the K concentration in the cell water (*i.e.* $\Delta K_i = 7\,\Delta K_0$). It is to be expected therefore that the same general relation would hold wherever K is penetrating into cells.

Evidence has been presented that the initial rise in potassium concentration resulting from injection is followed by a secondary rise, as if the potassium were coming out of the tissues again. In an effort to study this phenomenon many variations in experimental technique were tried, as listed in Table I. These included changes in the anion, the anesthetic, the injection site, kidney elimination, and pulmonary ventilation. None of these produced consistent changes in the course of the experiment.

Data from Cat 18 (with kidneys intact) suggest that part of the potassium comprising this secondary rise came from endogenous sources. During the first 90 minutes following injection, 23 mg. of K appeared in the urine and the plasma potassium was still at the height obtained by distributing 25 mg. of exogenous K throughout the extracellular space (30 per cent of the cat). The difference between the sum of these values and the 35 mg. of K injected is 13 mg. of K, which must have come from some endogenous source.

A release of liver potassium by adrenalin has been established (D'Silva, 1937; Houssay *et al.*, 1936) and injections of potassium are known to liberate adrenalin (Hazard, 1933; Katz and Katz, 1937). Injected potassium may therefore release additional endogenous liver potassium. This, however, would occur during the first 5 minutes after injection—too soon to explain the secondary increase discussed here.

In the literature may be found some further evidence of a potassium reappearance, as in the report of Winkler and Smith (1938). In the work of Houssay and Marenzi (1937), who injected KCl into normal dogs, there is a slight rise of potassium after 60 minutes. Also the sudden increase of plasma potassium which follows the administration of adrenalin and other substances (Houssay *et al.*, 1936) is succeeded by a later potassium rise. Keyes (1937) described such a rise in man 40 minutes after exercise. This increase suggests that, although 90 to 95 per cent of the injected potassium is temporarily absorbed rather rapidly by the tissues, a part of it later returns to the blood as if to seek more permanent storage elsewhere.

I am very grateful to Professor W. O. Fenn for helpful advice and criticism received during this work.

SUMMARY

1. When potassium is injected into the arteries or veins of cats, it diffuses rapidly into the tissues, its concentration in the

plasma reaching a minimum in 15 to 25 minutes. At this time only one-half of the injected potassium can be accounted for in the extracellular spaces of the body (30 per cent of the body weight), so that the remainder must have penetrated into some of the tissue cells. It is probably concentrated in some cells and absent from others.

2. After this minimum there is a secondary rise of potassium in the blood, reaching a maximum 40 minutes after injection, as if the potassium were temporarily stored in one location from which it moved later for more permanent storage elsewhere. Data from seventeen injections show that there is only one chance in 62 that this rise is not real.

BIBLIOGRAPHY

Bourdillon, J., *Am. J. Physiol.*, **120**, 411 (1937).

D'Silva, J. L., *J. Physiol.*, **90**, 303 (1937).

Fenn, W. O., and Cobb, D. M., *J. Gen. Physiol.*, **17**, 629 (1934); *Am. J. Physiol.*, **112**, 41 (1935).

Hazard, R., *Compt. rend. Acad.*, **197**, 1455 (1933).

Houssay, B. A., and Marenzi, A. D., *Rev. soc. argent. biol.*, **13**, 139 (1937).

Houssay, B. A., Marenzi, A. D., and Gerschman, R., *Rev. soc. argent. biol.*, **12**, 434 (1936).

Katz, G., and Katz, G., *J. Pharmacol. and Exp. Therap.*, **59**, 284 (1937).

Keyes, A., *Science*, **85**, 317 (1937).

Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., *J. Clin. Inv.*, **15**, 261 (1936).

Thaler, J. I., *Proc. Soc. Exp. Biol. and Med.*, **33**, 368 (1935).

Winkler, A. W., and Smith, P. K., *J. Biol. Chem.*, **124**, 589 (1938).

THE ISOLATION OF A MUCOPOLYSACCHARIDE FROM SYNOVIAL FLUID*

By KARL MEYER, ELIZABETH M. SMYTH, and
MARTIN H. DAWSON

(From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, and the Institute of Ophthalmology and the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York)

(Received for publication, January 30, 1939)

It has been known for a long time that synovial fluid on acidification yields a stringy precipitate, the so called synovial mucin, which redissolves in alkali to form a viscous solution. Much work has been done on the distribution and properties of this fraction and its production by special cells of the synovial lining. Even its formation in tissue cultures of synovial tissue has been demonstrated. (For literature see (2).)

Some authors have contended that the synovial fluid is a secretion of specific cells of the synovial lining; others have thought it to be a dialysate of the blood stream modified by products of degenerated tissue. In view of the importance and frequency of diseases such as rheumatic fever and arthritis, much attention has been devoted to the quantity and the viscosity of the fluid and the "mucin" isolated from it. It seems doubtful, however, whether the method of isolation of the "mucin" (precipitation with 2 per cent acetic acid) gives a quantitative yield of unaltered material.

No information was available about the chemical nature of synovial "mucin" other than data on the nitrogen and sulfur content and the appearance of a reducing substance after hydrolysis.

In order to isolate the substance responsible for the viscosity of the synovial fluid we have used a method similar to that employed in the isolation of chondroitinsulfuric acid (3), avoiding strong alkali and acid. A polysaccharide acid of high molecular weight was thus obtained from bovine synovial "mucin," possessing most of the viscosity of the starting material.

---

* A preliminary report of this work has been published (1).

It is obvious from the methods of preparation that the polysaccharide acid occurs in the fluid either free or united to protein in salt linkage only. Such a salt should dissociate in weakly alkaline solution, and a separation of the protein cation and its polysaccharide anion should be possible if the two constituents possess different solubilities. This proves to be the case. The synovial "mucin" can be separated into its protein and carbohydrate components by salting-out the protein in weak ammoniacal solution. The protein is precipitated by half saturation with ammonium sulfate and the carbohydrate can be obtained from the viscous supernatant solution by alcohol precipitation.

The polysaccharide acid consists of equimolar parts of hexosamine, hexuronic acid, and acetyl, the latter apparently as N-acetylglucosamine. The yield varied from 200 to 300 mg. per liter of synovial fluid. The concentration of polysaccharide calculated from the hexosamine content of the "mucin" was about 25 per cent greater than that accounted for by the isolated polysaccharide acid. The remaining hexosamine is apparently part of the protein moiety. The protein isolated by the salting-out method gave after several reprecipitations 1.5 per cent hexosamine on analysis. On the basis of the uronic acid determinations of the "mucin," the hexosamine content of the protein was calculated as 1.8 per cent instead of the 1.5 per cent found. In its hexosamine content as well as in its physical properties, the protein resembles serum globulin. The polysaccharide acid constitutes about 13 to 14 per cent of the "mucin" complex. It has been shown (4) that acid polysaccharides form true salts with the basic amino groups of proteins, which precipitate if the latter are ionized, *i.e.* in acid solution.

In addition to the hexosamine contained in the polysaccharide acid and the globulin fraction, a still larger amount of the hexosamine (about 60 per cent) found in the original synovial fluid is contained in fractions not accounted for by isolation. The hexosamine content of this residual fraction is in close agreement with the hexosamine content of normal serum, calculated on the basis of total solids. It is interesting to point out that this finding contrasts with the results obtained with aqueous and vitreous humor, where the polysaccharide isolated accounts for about 90 per cent of the total hexosamine in these fluids. Two possible explanations for the discrepancy may be offered: (1) the proteins found in synovial fluid may be the normal serum proteins, (2) they may result from inflammatory and traumatic processes.

In composition and rotation the polysaccharide acid of synovial fluid appears to be identical with the polysaccharide acid of vitreous humor, umbilical cord (5), and the mucoid phase of Group A hemolytic streptococcus (6). This is also borne out by the enzymatic hydrolysis of the new polysaccharide, by a specific enzyme obtained from pneumococcus (7),[1] into acetylglucosamine and glucuronic acid. The amount of reducing sugar liberated accounts for 96 to 100 per cent of the analytically determined component carbohydrates.

Whether the polysaccharide acid of synovial fluid from species other than cattle is identical with the one described is not definitely known at present. However, from 160 cc. of an exudate from an inflamed human knee joint, we isolated 53.9 mg. of an impure polysaccharide having properties similar to the bovine fractions. This polysaccharide was also hydrolyzed by the pneumococcus enzyme.

Experiments on the immunological properties of the polysaccharide acid indicate that it does not possess the properties of a hapten. All attempts to obtain antisera to the carbohydrate have failed, as have experiments designed to increase the antigenicity of synovial fluid (8). Attempts to produce precipitating sera against the polysaccharide of hemolytic streptococci by immunizing rabbits with Group A organisms according to Loewenthal's method (9) have also met with failure.

EXPERIMENTAL

Preparation of the Polysaccharide Acid—The synovial fluid was obtained in the slaughter-house by aspiration of the astragalotibial joints of freshly killed cattle, mostly 1 year-old steers.[2] Usually 2 to 5 liters were worked up in each lot. In some instances the fluid was filtered. The fluid was diluted with 2.5 volumes of distilled water and the protein salt of the polysaccharide was formed by adding 10 per cent acetic acid to give a concentration of 0.2 per cent. This concentration, instead of the 2 to 3 per cent recommended by others, is sufficient to precipitate all of the polysaccharide. After 18 hours at 0–5°, the fibrous and sticky precipitate was partly removed by a glass rod and the rest by filtration. It was washed several times with distilled water and brought into solution with 10 per cent calcium chloride. Sodium hydroxide was added from time to time to keep the solution just alkaline to phenolphthalein (pH 9). On being stirred mechanically, the material slowly goes into solution. From this solution the bulk of the protein was removed by repeated shaking with chloroform-amyl alcohol mixture (5, 10). When the solution had become almost clear, it was treated with 1.25 volumes of alcohol. The centrifuged precipitate was washed with alcohol and dissolved in 5 per cent sodium acetate solution. If the solution was turbid, the treatment with chloroform-amyl alcohol was repeated. A further amount of protein was then removed by adding an 8 per cent zinc acetate solution and neutralizing with normal sodium hydroxide. Care must be taken at this stage to work in dilute solution (about 0.5 per cent with respect to carbohydrate), as otherwise the polysaccharide itself precipitates as a zinc salt. To remove zinc and sodium acetate the centrifuged solution was again treated with alcohol acidified with acetic acid. The precipitate was dissolved in a minimum amount of water and reprecipitated with 5 volumes of glacial acetic acid or alcohol acidified with glacial acetic acid. The voluminous, gelatinous mass was removed by centrifuging, washed on a filter with alcohol, acetone, and ether, and dried over phosphorus pentoxide. The free acid thus isolated consists of white fibers of considerable tensile strength.

---

[1] We have obtained uniformly highly active enzyme preparations from autolyzed pneumococci. The enzyme has also been prepared from cultures of Group A hemolytic streptococci. This work will be reported in a separate communication.

[2] We wish to thank the management of Wilson and Company for their splendid cooperation.

The neutralized 0.25 per cent solution of one sample (No. 82) in 0.9 per cent sodium chloride had a viscosity, determined at 20° in an Ostwald viscosimeter, of 9.33 relative to 0.9 per cent sodium chloride.

The separation of the protein salt into the protein and carbohydrate fractions was carried out on a once reprecipitated sample, which after dehydration with alcohol gave the following analysis: Sample 76Ia, N 12.63, hexosamine 7.55, uronic acid 6.17, acetyl 1.74, hexuronic acid to hexosamine ratio 0.75, acetyl to hexosamine ratio 0.96.

The protein separated out on addition of an equal volume of saturated ammonium sulfate solution and on making it alkaline with strong ammonia water to pH 9. The precipitate was filtered off through folded filter paper. Sulfate was removed from the clear, viscous solution by adding solid barium acetate and acidifying with a small amount of acetic acid, and the supernatant was treated with 2 volumes of alcohol. The precipitate was extracted with 5 per cent sodium acetate solution; the solution cleared by zinc hydroxide precipitation and again treated with alcohol. After one reprecipitation from alcohol, Sample 78II was obtained (Table I). The ammonium sulfate precipitate containing the protein fractions was taken up in water, neutralized, and fractioned by ammonium sulfate solution. Four fractions were obtained. For analysis the solutions were precipitated with 5 per cent trichloroacetic acid, washed three times with 2 per cent trichloroacetic acid, and dried.

Nitrogen	Hexosamine
13.4	1.5
14.4	1.7
13.2	1.3
14.0	1.3

Analysis of the polysaccharide fractions is shown in Table I. The analytical methods have been described previously (5, 11).

The high ash content is of special importance when one is considering whether the polysaccharide is originally, all or in part, a sulfuric acid ester. It has been claimed that the "mucin" of synovial fluid is a protein compound of chondroitinsulfuric acid (2). Analysis of Sample 69 (38 mg.) gave no volatile sulfur and in the ash 0.47 per cent sulfur. (The sulfur content of chondroitinsulfuric acid prepared by a similar method varied between 5.6 and 6.1 per cent, half of which appears as volatile sulfur.) Phosphorus was present in traces too small to determine. The ash content is probably explained by the difficulty experienced in washing the extremely voluminous slimy precipitates.

Another indication of the absence of appreciable quantities of chondroitinsulfuric acid is found in the rotation of the hexosamine hydrochloride isolated from the polysaccharide. 0.5029 gm. of a preparation containing 33.1 per cent hexosamine was hydrolyzed with hydrochloric acid and the amino sugar hydrochloride isolated as described previously (5). The 0.1765 gm. of hexosamine hydrochloride isolated accounted for 88.1 per cent of the amount found by analysis. The nitrogen content was 6.40 per cent (theoretical 6.47), and the rotation at equilibrium was +70.1° (theoretical for *d*-glucosamine hydrochloride, +72.5°). The hexosamine was therefore *d*-glucosamine. In the presence of chondroitinsulfuric acid, the isolated hexosamine would have shown a

TABLE I

Analysis of Synovial Fluid Polysaccharide Corrected for Ash and Moisture

Sample No.	Nitrogen	Hexosamine	Uronic acid	Acetyl	Acid equivalent weight	$[\alpha]_D$	Moisture	Ash	Equivalents per equivalent weight			
									Nitrogen	Hexosamine	Uronic acid	Acetyl
	per cent	*per cent*	*per cent*	*per cent*		*degrees*	*per cent*	*per cent*				
61	3.69	38.2	41.3	9.4	454	−68.8	3.28	3.97	1.19	0.97	0.97	1.00
67	3.68	41.8	44.1	10.6	511	−71.9	8.73	2.08	1.34	1.19	1.16	1.26
69	3.59	43.7	50.7	10.3	477	−73.8	5.60	4.95	1.22	1.16	1.25	1.15
77AI	3.36	34.5	38.4	10.5	401	−78.2	3.50	4.14	0.96	0.77	0.79	0.98
78II	3.21	36.0	35.9	10.5	492	−69.9	5.61	2.50	1.12	0.99	0.91	1.20
82	3.00	40.3	44.5	10.3	520	−77.4	5.10	2.90	1.11	1.17	1.19	1.24

TABLE II

Enzymatic Hydrolysis of Polysaccharide from Synovial Fluid

The concentration of the polysaccharide was 0.5 per cent. Hydrolysis shows 96 per cent of the theoretical amount calculated from reducing values for acetylglucosamine and glucuronic acid.

Concentration of enzyme in mixture	Glucose (theoretical = 0.585 mg.) from 1 mg. polysaccharide (Hagedorn-Jensen method) corrected for controls		
	2 hrs.	17 hrs.	41 hrs.
per cent	*mg.*	*mg.*	*mg.*
0.1	0.531	0.563	0.563
0.05	0.522	0.558	0.568
0.025	0.434	0.556	0.558

rotation intermediate between glucosamine and chondrosamine, as shown previously (5).

The enzymatic hydrolysis of the polysaccharide from synovial fluid is illustrated in Table II.

In Fig. 1 is shown a comparison of the hydrolysis of the polysaccharides from synovial fluid and from Group A hemolytic streptococci[3] with two dilutions of enzyme. The enzyme employed in this experiment was prepared from a non-type-specific variant of Type II pneumococcus. At the intervals indicated in the graph 0.2 cc. was pipetted out and reducing sugar determined by the Hagedorn-Jensen method. Both the enzyme and substrates were incubated in buffer solution separately and the very small amount of reduction in these blanks subtracted. The course of the hydrolysis for the two polysaccharides runs parallel within the limits of error of the method. This together with the similarity in the chemical and physical properties of the substance indicates their identity.

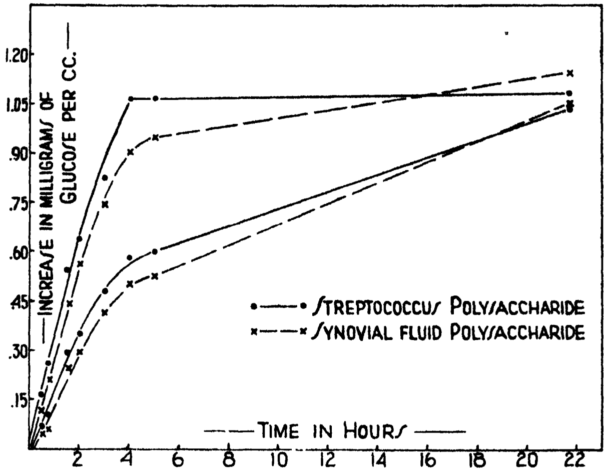


FIG. 1. Comparison of the rate of hydrolysis of polysaccharides from synovial fluid and from Group A hemolytic streptococci. The substrate concentration was 0.25 per cent and the enzyme concentrations 0.025 and 0.005 per cent.

---

[3] The streptococcus polysaccharide was a sodium salt obtained from Dr. Kendall.

The hexuronic acid present in the polysaccharides of vitreous humor and umbilical cord had been demonstrated as glucuronic acid by the isolation of its oxidation product saccharic acid (5). From the synovial polysaccharide the attempt was made to isolate glucuronic acid after enzymatic hydrolysis of the polysaccharide with the pneumococcus enzyme. 0.600 gm. of polysaccharide was incubated in the presence of toluene for 66 hours with 10 mg. of a purified enzyme preparation (as flavianate) from Type II pneumococcus. The reducing value at that time indicated that the polysaccharide had been hydrolyzed about 100 per cent. Glucuronic acid was precipitated as the basic lead salt. From this fraction the thiosemicarbazone was prepared by Neuberg's method (12), having a melting point of 223–224° (m.p. found by Neuberg 223°). According to Neuberg the thiosemicarbazone is typical for glucuronic acid.

Acetylglucosamine was not isolated. An attempt was made to follow the hydrolysis of the polysaccharide by the pneumococcus enzyme by the quantitative acetylglucosamine determination of Morgan and Elson (13). Neither the polysaccharide nor the enzyme gave a blank value after incubation, while the mixture of the two gave an increasingly intense color after incubation. However, it has not been possible to get satisfactory data by this method.

SUMMARY

From the stringy precipitate which settles out from synovial fluid after acidification, the so called synovial "mucin," a polysaccharide acid has been prepared. This polysaccharide consists of equimolar parts of a hexosamine, a hexuronic acid, and acetyl. The hexosamine has been demonstrated as *d*-glucosamine and the uronic acid as glucuronic acid. The polysaccharide acid is apparently identical with the polysaccharides previously isolated from vitreous humor and umbilical cord, and with that present in Group A hemolytic streptococcus. All four polysaccharides contain the same components in equimolar amounts, have a similar optical rotation, and are hydrolyzed at the same rate by a specific enzyme. The protein component of the carbohydrate-protein complex has been obtained by precipitation with ammonium sulfate in weakly alkaline solution. It appears to

be a globulin which apparently forms an insoluble salt with the acid polysaccharide on acidification.

We wish to acknowledge the assistance of Mr. Arthur Stempel and Miss Eleanor Chaffee during the latter part of this work.

BIBLIOGRAPHY

1. Meyer, K., Smyth, E. M., and Dawson, M. H., *Science*, **88**, 129 (1938).
2. Kling, D. H., The synovial membrane and the synovial fluid, Los Angeles (1938).
3. Meyer, K., and Smyth, E. M., *J. Biol. Chem.*, **119**, 507 (1937).
4. Meyer, K., Palmer, J. W., and Smyth, E. M., *J. Biol. Chem.*, **119**, 501 (1937).
5. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **114**, 689 (1936).
6. Kendall, F. E., Heidelberger, M., and Dawson, M. H., *J. Biol. Chem.*, **118**, 61 (1937).
7. Meyer, K., Dubos, R., and Smyth, E. M., *J. Biol. Chem.*, **118**, 71 (1937).
8. Swift, H. F., and Schultz, M. P., *J. Exp. Med.*, **63**, 703, (1936).
9. Loewenthal, H., *Brit. J. Exp. Path.*, **19**, 164 (1938).
10. Sevag, M. G., *Biochem. Z.*, **273**, 419 (1934).
11. Palmer, J. W., Smyth, E. M., and Meyer, K., *J. Biol. Chem.*, **119**, 491 (1937).
12. Neuberg, C., *Ber. chem. Ges.*, **33**, 3322 (1900).
13. Morgan, W. T. J., and Elson, L. A., *Biochem. J.*, **28**, 988 (1934).

THE VITAMIN C CONTENT OF HUMAN URINE AND ITS DETERMINATION THROUGH THE 2,4-DINITROPHENYLHYDRAZINE DERIVATIVE OF DEHYDROASCORBIC ACID*

By JOSEPH H. ROE and JAMES M. HALL

(*From the Department of Biochemistry, School of Medicine, George Washington University, Washington*)

(Received for publication, February 6, 1939)

It is well known that the determination of ascorbic acid in urine by titration with 2,6-dichlorophenol indophenol in acid solution is not a specific method. Various interfering substances have been reported, such as sulfhydryl compounds (1), thiosulfate (2), and pyridium (medication) (3). The same limitations apply to other oxidation-reduction methods, except when the reducing substance is determined before and after oxidation by ascorbic acid "oxidase" (4). The latter procedure is unsatisfactory for urine for two reasons: It has been found that the "oxidase" is not specific for the oxidation of ascorbic acid (5); and the enzyme may not be effective in oxidizing the vitamin, as shown by Bezssonoff and Vertruyen (6) who found that ascorbic acid "oxidase" prepared from pumpkin pulp did not act upon ascorbic acid in human urine, spinal fluid, and cow's milk.

To remove interfering reducing and colored substances from urine, Emmerie and van Eekelen (7) used mercuric acetate as a precipitant of the interfering material. Later, van Eekelen and Emmerie (8) showed that mercuric acetate precipitation must be carried out rapidly, and excesses of the reagent must be avoided to prevent irreversible oxidation of ascorbic acid. Reducing the severity of treatment minimizes, but does not remove, the error from this procedure. Furthermore, when mercuric acetate is used,

---

* Aided by a grant from the Committee on Scientific Research, American Medical Association.

We are indebted to Merck and Company for a generous supply of pure ascorbic acid.

the dehydroascorbic acid is reduced and mercury is removed by introducing H_2S. The latter procedure may give rise to a plus error. Scarborough and Stewart (9) showed that urine contains substances other than ascorbic acid capable of slow reduction with H_2S to indophenol-reducing compounds.

The ordinary indophenol, visual titration method has been greatly improved by Evelyn, Malloy, and Rosen (10), who make a photoelectric measurement of the amount of dye decolorized and take readings 5, 10, 20, and 30 seconds after mixing the dye and urine, extrapolating to zero time to get a reading which eliminates approximately the error due to other reducing substances. This procedure has introduced the greatest advance made in indophenol titration, but, as stated by its authors (10), "This method is admittedly empirical and cannot therefore be expected to give more than approximate results, but the errors are far less serious than those of the ordinary visual titration . . ."

In view of the limitations of the reduction methods, it seemed important to devise a procedure based upon some other principle than oxidation-reduction. Such a method has been developed. In this method, ascorbic acid is oxidized to dehydroascorbic acid by passing the urine through norit, and the dehydroascorbic acid is separated as a 2,4-dinitrophenylhydrazine derivative. This derivative, which is an osazone with dinitrophenylhydrazine groups attached to carbon atoms 2 and 3 (11), is dissolved in HCl containing $SnCl_2$ and boiled under 15 pounds pressure. By this treatment, the nitro groups are reduced, giving a colorless substance, and the compound is hydrolyzed, liberating dehydroascorbic acid; the latter forms furfural when boiled with HCl containing $SnCl_2$ (12). The furfural is determined colorimetrically by the aniline acetate method (12).

This procedure has been applied widely to the determination of ascorbic acid in plant and animal tissues. In this paper, we are reporting the results of analyses of 57 freshly voided samples of urine from 50 normal human subjects by the proposed method, and by indophenol titration. The procedures used were as follows:

Osazone-Furfural Method

To 50 cc. of urine, add 5 cc. of glacial acetic acid and 2 gm. of metaphosphoric acid. After the latter has dissolved, add 5 gm.

of norit and shake vigorously. Filter. The filtrate must be perfectly colorless; if it is not, add more norit and filter again. Place 1, 2, and 3 cc., of the norit filtrate in 15 cc. conical Pyrex centrifuge tubes with a mark at 7 cc.

Prepare standards as follows: Dissolve 25 mg. of pure ascorbic acid in 25 cc. of 10 per cent acetic acid containing 4 per cent of metaphosphoric acid. Dilute 2 cc. of this solution to 100 cc. with the same acetic-metaphosphoric acid solution. 1 cc. of this solution contains 0.02 mg. of ascorbic acid. Add norit in excess (5 gm.) and shake vigorously. Filter. Place 1, 2, and 3 cc. in 15 cc. conical Pyrex centrifuge tubes with a mark at 7 cc. Set up a series of these tubes according to needs.

To the unknown and standard tubes add 2 volumes of saturated, purified 2,4-dinitrophenylhydrazine[1] in N HCl. This reagent must be measured with quantitative precision. It should be added to the standards and the unknowns at approximately the same time; hence, place all unknown and standard solutions in the centrifuge tubes before adding the reagent. Let stand for 15 to 24 hours for reaction and crystallization.

On the following day, centrifuge all tubes at a speed which will pack the crystalline 2,4-dinitrophenylhydrazine derivative in the bottom of the tubes. It may be necessary to break up the clusters of crystals with a stirring rod to make them pack satisfactorily in the centrifuge tubes.

After successful centrifugation, decant the supernatant fluid carefully, and set the tubes in a beaker in an inverted position to allow drainage for several minutes. Wash the precipitates by delivering forcefully upon the mat of crystals 5 cc. of 10 per cent formic acid. This is done by blowing the washing solution from a pipette. The mat of osazone crystals must be thoroughly broken up. Centrifuge, and drain carefully.

To the mat of crystals in each of the centrifuge tubes, add 2 cc. of 12 per cent HCl containing 10 per cent of $SnCl_2$. Place in a beaker of boiling water and boil until the crystals are dissolved. It will be necessary to remove the centrifuge tubes from the beaker with forceps from time to time, and tilt each tube so as to bring the hot acid solution in contact with crystalline material adhering to the sides. Finally, when all of the osazone crystals have been dissolved, place the tubes containing the standards and unknowns in an autoclave and heat in an atmosphere of steam for 30 minutes at 15 pounds pressure. Cool, and remove the tubes to a rack. To each tube add 2 cc. of glacial acetic acid and mix; then add 3 cc. of alcoholic aniline (2 parts of redistilled aniline to 5 parts of ethyl alcohol). Keep the alcoholic aniline in a dark bottle in a refrigerator. Remove from the refrigerator and add while cold. Make up to exactly 7 cc. with H_2O. Mix by inversion and place immediately in cold water. After 5 minutes, place the tubes in a rack, and let stand for an additional 10 minutes. At all times after adding aniline keep the tubes away from exposure to light as much as possible. Finally, compare in a colorimeter, using the most appropriate standard.

---

[1] 2,4-Dinitrophenylhydrazine is conveniently recrystallized from ethyl acetate solution.

Calculation—

$$\frac{S}{U} \times S_1 \times \frac{1000}{\text{cc. norit filtrate}} \times 1.1 = \text{mg. ascorbic acid per liter urine}$$

where, S = reading of standard, U = reading of unknown, and S_1 = mg. of ascorbic acid in the standard used.

All measurements in this procedure must be rigidly accurate. The reaction yielding the osazone of dehydroascorbic acid is a slow one and equilibrium is not reached in 24 hours. Exactly 2 volumes of 2,4-dinitrophenylhydrazine solution must be added and the same temperature conditions must be maintained for the standards and the unknowns. The quantitative character of the method is dependent upon identical treatment of standards and unknowns.

From an occasional urine a slight brown color may be obtained when the osazone crystals are dissolved, which will later interfere in the pink color that is produced. Such urines should be passed a second time through additional norit, which adsorbs the interfering substances. The maximum absorption of the pink color formed by the reaction of furfural with aniline is at 520 mμ.[2] A green filter (Wratten No. 58) may therefore be used to advantage if this interfering color appears. It is best to avoid this interference, however, by a more vigorous treatment of the urine with norit.

---

[2] We are indebted to Mrs. A. P. Bradshaw of the United States Department of Agriculture for the preparation of spectrophotometric absorption curves.

Indophenol Titration Method

The urines studied by us were either titrated immediately after being voided, or metaphosphoric and acetic acids were added and the titrations were carried out within 2 hours after collection. 10 cc. of urine were placed in a white casserole and 2 cc. of glacial acetic acid were added. Indophenol solution, standardized against the same standard of pure ascorbic acid as used in the osazone-furfural method, was titrated into the urine. An end-point lasting 10 seconds was taken as the end of the titration.

Discussion of Osazone-Furfural Method

The specificity of this method is dependent upon the absence from urine of furfural precursors capable of coupling with 2,4-dinitrophenylhydrazine in amounts that will interfere. Of the latter substances, glucuronic acid and pentoses must be considered. Glucuronic acid does not produce enough hydrazone to interfere until amounts in excess of 5 mg. per cc. are present. The normal glucuronic acid content of urine is 300 to 660 mg. per 24 hour specimen (13), or approximately 0.3 to 0.6 mg. per cc. Hence interference from glucuronic acid will not occur in normal urines, and may not be expected until at least 10 times the normal amount is present.

We determined the furfural precursors in norit filtrates of twenty urines and found values ranging from 0.02 to 0.23 mg. per cc. as xylose. These values represent principally pentose content, as glucuronic acid and hexoses have a comparatively small furfural-producing capacity (14). Xylose does not yield furfural-producing hydrazone until a concentration in excess of 1 mg. per cc. is present. Therefore interference from pentoses will not occur in normal urines and may not be expected until about 5 times the normal amount is present.

If one suspects abnormal amounts of furfural precursors in the urine, the total amount of these substances may be determined as follows: To 0.1 cc. of norit filtrate and 0.1 cc. of xylose standard solution containing 0.02 mg. of xylose in test-tubes are added 2 cc.

of 12 per cent HCl. Boil in a water bath for 15 minutes. Cool and add 1 cc. of 1 per cent $SnCl_2$ to each tube. From here on, treat with acetic acid and alcoholic aniline the same as above with the autoclaved osazone solutions.

We have repeatedly added ascorbic acid to urines containing vitamin C and to urines not containing this vitamin, and have obtained recoveries ranging from 95 to 105 per cent by this method. The procedure therefore appears entirely specific for the determination of vitamin C in urine.

Results of Comparative Studies

The results of our comparative analysis of 57 samples of urine from 50 normal subjects are shown in Table I. Our subjects were medical students and laboratory workers, of whom forty-six were males and four were females. Where values above zero were obtained by the osazone-furfural method, the corresponding indophenol titration values range from 0 to 297 per cent higher. In the last column of Table I are data obtained by taking the difference between the results by the two methods. We interpret these values as representing the non-ascorbic acid indophenol-reducing substances of normal urine. They are of considerable significance. They indicate that indophenol titration values up to 29 mg. per liter represent an unknown amount of ascorbic acid; and that one cannot be sure that any part of an indophenol titration value is true ascorbic acid except that found in excess of 29 mg. per liter.

The results in Table I are expressed as mg. per liter. For purposes of comparison these figures may be considered as representing 24 hour excretion values. The vitamin C content of the urine of normal adults is usually stated to be 10 to 30 mg. per 24 hour sample (15, 16) when determined by indophenol titration. These figures have little significance in view of our finding of 0 to 29 mg. of non-ascorbic acid indophenol-reducing substances per liter of urine.

These results have an important bearing upon the procedures used to determine the human requirements of vitamin C. In their early work, Harris and his associates (15) used a urinary "resting level" of 15 mg. per 24 hours, determined by indophenol titration, as indicating the border line between sufficiency and inadequacy of vitamin C intake, combining this criterion with the requirement that the subject must also fail to excrete increased amounts of vitamin C after administration of a 700 mg. dose of this vitamin

TABLE I

Ascorbic Acid per Liter of Urine

Indophenol titration method	Osazone-furfural method	Non-ascorbic acid indophenol-reducing substances	Indophenol titration method	Osazone-furfural method	Non-ascorbic acid indophenol-reducing substances
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
33.0	33.0	0	15.5*	5.5	10.0
34.0	34.0	0	18.7*	8.6	10.1
55.0	55.0	0	10.4	0	10.4
74.0	74.0	0	58.0†	47.0	11.0
6.0	5.0	1.0	39.0†	28.0	11.0
67.0	66.0	1.0	41.0†	30.0	11.0
84.9	82.0	2.9	42.0†	31.0	11.0
38.0	35.0	3.0	27.0	16.0	11.0
39.5	35.6	3.9	91.0	79.0	12.0
43.2	39.2	4.0	13.0	0	13.0
41.3	36.5	4.8	14.0	0	14.0
5.0	0	5.0	24.9	10.7	14.2
5.0	0	5.0	28.3	14.0	14.3
14.6	9.6	5.0	15.0	0	15.0
96.4	91.1	5.3	16.0	0	16.0
6.0	0	6.0	22.5	6.1	16.4
11.0	5.0	6.0	27.9	11.1	16.8
30.5	23.5	7.0	137.2	120.1	17.1
14.3*	7.1	7.2	29.7	12.2	17.5
18.2	10.4	7.8	18.0	0	18.0
12.7	4.3	8.4	34.3	16.2	18.1
31.8	22.9	8.9	35.1	17.0	18.1
19.0*	10.1	8.9	18.7	0	18.7
86.7	77.3	9.4	19.0†	0	19.0
21.5	11.8	9.7	28.6	9.6	19.0
15.2	5.3	9.9	20.0	0	20.0
10.0	0	10.0	47.0	25.0	22.0
23.0	13.0	10.0	36.6	13.1	23.5
			29.0	0	29.0

* Same subject.

† Same subject.

to show unsaturation. Largely as a result of the studies of Harris and coworkers, a urinary vitamin C content of 10 to 30 mg. per 24 hours, by indophenol titration, has been taken as indicating that there is an adequate intake of vitamin C. Our studies show that when a value of 10 to 30 mg. per liter is obtained by indophenol titration, there may be a small amount of vitamin C in the urine, but often there is none; and that, if the demonstration of ascorbic acid in urine is to be taken as a criterion of adequacy of intake, a border line urinary value of less than 29 mg. per liter by indophenol titration has no significance.

Our findings are confirmatory of the procedure of Widenbauer (17) for determining the human vitamin C requirement. Widenbauer places the subject on a uniform diet containing very little vitamin C for a preliminary period of several days. The urine is titrated with indophenol and the average daily content is taken as a blank to be deducted from the daily excretion when test doses are given. Widenbauer's blank values obtained in this way probably correspond to the amounts of non-ascorbic acid indophenol-reducing substances of our data, and the desirability of establishing such values is confirmed by our studies.

By the osazone-furfural method analysis of 4 cc. of a solution containing 0.005 mg. of ascorbic acid per cc. gave a satisfactory color and quantitative recoveries, when compared with 1 cc. of solution containing 0.02 mg. Taking into consideration the lower limits of the sensitivity of this method, we can say that the zero values of our data must represent less than 5 mg. per liter, and are very close to, if not true, zero values. It is therefore of considerable interest that fourteen of our 50 subjects, who were apparently on an adequate diet, showed no vitamin C present in the urine by our technique. These findings are in agreement with the report of Evelyn, Malloy, and Rosen (10), who noted that "24 hour ascorbic acid excretions of less than 5 mg. are often found in normal healthy individuals on adequate well balanced diets."

SUMMARY

1. A new method for the determination of ascorbic acid has been developed. Ascorbic acid is oxidized with norit and the dehydroascorbic acid is separated as a 2,4-dinitrophenylhydrazine derivative. This derivative is decomposed by boiling with HCl containing $SnCl_2$ with the formation of furfural. The furfural is determined colorimetrically by the aniline acetate method.

2. A comparative study of results by this method and by indophenol titration was made upon the urine from 50 normal human subjects. The indophenol titration gave values ranging from 0 to 297 per cent higher than those by the proposed method.

3. The urine from fourteen of our 50 subjects who were apparently on an adequate diet showed no vitamin C by the new method.

BIBLIOGRAPHY

1. Emmerie, A., *Biochem. J.*, **28**, 268 (1934).
2. van Eekelen, M., *Acta brev. Neerl. Physiol., Pharmacol., Microbiol.*, **4**, 137 (1934).
3. Gannon, C. F., and McGovern, T., *Proc. Soc. Exp. Biol. and Med.*, **38**, 267 (1938).
4. Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, **110**, 559 (1935).
5. Snow, G. A., and Zilva, S. S., *Biochem. J.*, **32**, 1926 (1938).
6. Bezssonoff, N., and Vertruyen, H., *Compt. rend. Soc. biol.*, **128**, 407 (1938).
7. Emmerie, A., and van Eekelen, M., *Biochem. J.*, **28**, 1153 (1934).
8. van Eckelen, M., and Emmerie, A., *Biochem. J.*, **30**, 25 (1936).
9. Scarborough, H., and Stewart, C. P., *Biochem. J.*, **31**, 2232 (1937).
10. Evelyn, K. A., Malloy, H. T., and Rosen, C., *J. Biol. Chem.*, **126**, 645 (1938).
11. Roe, J. H., *Proc. Soc. Exp. Biol. and Med.*, **37**, 465 (1937).
12. Roe, J. H., *J. Biol. Chem.*, **116**, 609 (1936).
13. Maughan, G. B., Evelyn, K. A., and Browne, J. S., *J. Biol. Chem.*, **126**, 567 (1938).
14. Youngburg, G. E., *J. Biol. Chem.*, **73**, 599 (1927).
15. Abassy, M. A., Harris, L. J., Ray, S. N., and Marrack, J. R., *Lancet*, **2**, 1399 (1935).
16. Magnusson, A. E., and Osterberg, A. E., *Proc. Staff Meetings Mayo Clin.*, **13**, 700 (1938).
17. Widenbauer, F., *Klin. Woch.*, **16**, 600 (1937).

THE AMINO ACID COMPOSITION OF ERYTHROCYTE POSTHEMOLYTIC RESIDUE OF FIVE MAMMALIAN SPECIES*

By ELIOT F. BEACH, BETTY NIMS ERICKSON, SAMUEL S. BERNSTEIN, HAROLD H. WILLIAMS, and ICIE G. MACY

(From the Research Laboratory of the Children's Fund of Michigan, Detroit)

(Received for publication, February 3, 1939)

During a series of investigations concerning the chemical structure of the red blood cell, a method has been developed in this laboratory (1) for the preparation of a posthemolytic residue containing only small amounts of hemoglobin. By this procedure it was possible to prepare from the bloods of several mammalian species, quantities of this material sufficient to permit detailed lipid and protein analyses. Previous studies (2) have demonstrated that the product is a protein-lipid complex and that, while the total lipid content of the material varies between 10 and 25 per cent in different species, the lipid pattern is strikingly similar in all of them. The major lipid constituent is phospholipid, of which more than 50 per cent is cephalin. Nitrogen, lipid, and ash analyses indicated that 90 to 95 per cent of the posthemolytic residue could be accounted for as ash and a lipid-protein complex which presumably forms the supporting structure of the red blood cell and which has been called "erythrocyte stroma." Analyses of these posthemolytic residues have been extended to include the protein moiety.

Posthemolytic residues were prepared from washed erythrocytes of normal beef, sheep, horse, hog, and human bloods[1] and were analyzed for their content of those amino acids for which reliable methods are available. The work was conducted in an attempt to discover any chemical differences in the protein fractions of the species studied and whether the same similarity of pattern which characterized the lipid fractions is to be found in the protein components.

---

* This paper was presented before the American Society of Biological Chemists at the Thirty-second annual meeting at Baltimore, March 30 to April 2, 1938.

[1] The authors wish to thank Mr. Harvey Merker, Dr. L. T. Clark, and Dr. A. S. Schlingman, of Parke, Davis and Company, Detroit, for their cooperation in securing the equine and human bloods.

EXPERIMENTAL

The posthemolytic residues were prepared from washed erythrocytes as described in a previous paper (1). The samples were dried *in vacuo*, the lipid fractions removed by repeated extractions with ethyl alcohol and ether, and the residue dried at 60–70° in a vacuum oven. Nitrogen analyses were made by the manometric micromethod of Van Slyke (3) except in instances in which sufficient material permitted a macro-Kjeldahl determination. Sulfur was determined gravimetrically as barium sulfate, after preliminary oxidation in the oxygen bomb.

Basic Amino Acids[2]—The hexone bases were determined by the method of Block (4). Arginine was calculated from the weight of its flavianate derivative. Lysine was determined through its picrate. Histidine was determined by isolation of histidine nitranilate, described by Block (5), from the histidine fraction, which had been purified by the mercury and copper salt procedure. The results were not corrected for any solubility losses which might have occurred during the procedure; therefore, the values reported may be considered minimal.

Tyrosine and Tryptophane—Tyrosine and tryptophane were estimated by the colorimetric procedure of Folin and Marenzi (6) with the following modifications. Hydrolysis was effected by heating with 20 per cent sodium hydroxide in the autoclave at 2 atmospheres for 6 hours as described by von Deseö (7). A tyrosine standard solution was used for comparison with the tryptophane color and the tryptophane value calculated from the formula of von Deseö (7) which corrected the original error[3] in the formula of Folin and Marenzi (6).

---

[2] Appreciation is extended Dr. Richard J. Block for so kindly accepting one of the authors (E. F. B.) in his laboratory to learn the methods of determining the basic amino acids and for his helpful advice.

[3] In the procedure of Folin and Marenzi (6) the tryptophane color in the unknown is compared with the same blue color developed in a tyrosine

Cystine—Cystine was determined by the method of Graff, Maculla, and Graff (8). The results are based upon the nitrogen content of the cysteine cuprous mercaptide isolated from small samples of the protein hydrolysates. This method has been found satisfactory; it yields easily reproducible results and it has given cystine values for edestin and casein in good agreement with those obtained by other reliable methods (Table I). By the Graff, Maculla, and Graff method, cystine may be recovered quantitatively from pure solution and also when added to hydrolysates of known cystine content.

Methionine—Methionine was determined by the latest method of Baernstein (9), which depends on the titration of homocysteine (formed during the reaction of methionine with hydriodic acid) with sodium tetrathionate. The earlier method of Baernstein, which depends upon the determination of volatile iodide liberated during hydriodic acid digestion, was not satisfactory when applied to the protein hydrolysates. The values given by the volatile iodide method were high and apparently were influenced by some substances other than methionine which yield a volatile iodide under the conditions of the analysis. Possibly this is due to small amounts of lipid not removed by alcohol and ether extraction.

Results

The results of the analyses of stromal protein for seven amino acids and nitrogen and sulfur in the lipid-extracted posthemolytic residues of five species are shown in the average values presented in Table I. One fact is immediately obvious: the proteins of the posthemolytic residues are similar in amino acid composition. Histidine in the different species' stromal proteins varied from 1.9 to 2.3 per cent, arginine from 4.6 to 5.3 per cent, lysine from 3.5 to 3.8 per cent, tyrosine from 2.7 to 2.9 per cent, and tryptophane from 1.1 to 1.2 per cent. The average nitrogen content of preparations from different species varied from 12.9 to 14.0 per cent and the sulfur from 0.71 to 0.78 per cent. Greater variations were

---

standard with the phenol reagent. The tryptophane unknown is calculated as tyrosine and then, according to Folin and Marenzi (6), it should be multiplied by 0.843 to convert it to the correct quantity of tryptophane. However, von Deseö (7) indicates that the tyrosine equivalent should be multiplied by 1/0.843 to convert it to the correct quantity of tryptophane.

observed in cystine and methionine content. Hog stromal protein showed a high cystine content, 1.11 per cent, while those of the other four species varied from 0.78 to 0.95 per cent. Beef stromal protein exhibited a low methionine content, 1.5 per cent, and sheep the high methionine content of 2.1 per cent, while the

TABLE I

Per Cent Nitrogen, Sulfur, and Amino Acid Composition of Lipid-Extracted Posthemolytic Residue

	Histidine	Arginine	Lysine	Tyrosine	Tryptophane	Cystine	Methionine	Total N*	Total S
Beef	1.9	5.1	3.5	2.9	1.2	0.78	1.5	13.8	0.708
Sheep	2.3	5.3	3.5	2.9	1.1	0.95	2.1	14.0	0.784
Horse	1.9	4.9	3.8	2.9	1.1	0.85	1.9	12.9	0.715
Hog	2.1	4.6	3.5	2.8	1.2	1.11	1.7	13.1	0.716
Human	2.1	4.8	3.8	2.7	1.2	0.90	1.8	13.0	0.713
Horse and sheep (from Jorpes (10))	2.6†	5.8‡		3.0	1.5			14.75	
Edestin									
Authors'	1.5	14.5	1.8	4.2	1.3	1.10	2.52	17.1	
Literature	2.1	15.6	2.1	4.3	1.4	1.27	2.20	18.7	
	(4)	(4)	(4)	(6)	(6)	(11)	(9)		
Casein									
Authors'	1.6	3.6	6.2	6.2	1.4	0.30	3.04	15.1	
Literature	1.8	3.8	6.3	6.6	1.4	0.30	3.10	15.7	
	(12)	(12)	(12)	(13)	(13)	(11, 14)	(9)		
						0.22			
						(19)			

The numbers in parentheses refer to the bibliography. All of the analytical values reported are averages of determinations run in duplicate or triplicate on single preparations except: in beef stromata, two preparations varied from 1.7 to 2.0 in histidine, and three preparations ranged from 4.9 to 5.3 in arginine, 3.3 to 3.7 in lysine, no variation in tyrosine, and 1.15 to 1.26 in tryptophane; and in human stromata, three preparations varied from 4.8 to 4.9 in arginine and two preparations did not vary in tyrosine or tryptophane.

* Nitrogen values are averages of determinations on two to four preparations in each of the species.

† Histidine obtained by the Pauli reaction.

‡ Arginine obtained by Sakaguchi and Van Slyke methods.

other three species gave approximately equal quantities of methionine, midway between the two extremes. Together, the cystine and methionine contents account for 85 to 95 per cent of the total sulfur. Among the stromal proteins the differences in cystine and methionine content are proportionately greater than with respect to any of the other amino acids. However, it is not possible, on the basis of a limited number of samples, to postulate a significant species variation.

These values for the amino acid composition of the lipid-extracted posthemolytic residue, with respect to arginine, histidine, tyrosine, and tryptophane, may be compared with those reported by Jorpes (10) and included in Table I. The values presented herein are similar but are all consistently lower. Two factors may account largely for the discrepancy: Jorpes' lipid-free posthemolytic residue contained about one-tenth more nitrogen than those prepared for this study; furthermore, he employed the Sakaguchi and Van Slyke methods for determining arginine, and the Pauli reaction for histidine. These methods would be expected to yield somewhat higher values for the basic amino acids than does the Block method which depends upon the isolation of the bases. Considering these facts, there is agreement between the values obtained and those of Jorpes. On the basis of the analytical values obtained for the basic amino acids, Jorpes' finding that the protein moiety of the stroma is not of strongly basic character is confirmed.

Previous analyses of the posthemolytic residue preparations (1) have shown that a considerable amount of organic iron is present and that this iron, calculated as hemoglobin, could account for as much as 5 to 10 per cent contamination by hemoglobin. At present it is believed that at least a portion of the iron may be a component of the true posthemolytic residue proper (10) and not present as hemoglobin contamination. If most of the organic iron does represent hemoglobin contamination, then the values reported for histidine and lysine, which are found in large amounts in hemoglobin, are somewhat high for the true stroma.

To determine the reliability of the procedures followed in this laboratory, amino acid analyses of casein[4] and of edestin have been carried out by the methods employed on the posthemolytic residue protein. The values obtained are presented in Table I and are compared with those reported in the literature.

---

[4] The casein was purchased from Eimer and Amend, prepared according to the method of Hammarsten.

In Table II the values for the basic amino acids contained in lipid-extracted stroma are compared with those found in the literature for serum orosin, hemoglobin, and cattle fibrin. The distribution of basic amino acids in the stromal protein preparation is obviously different from that of hemoglobin and cattle fibrin but possesses some similarity to Block's serum orosin. It differs from the serum protein, however, in possessing a lysine content of 3.6 per cent, about one-half as much as that found in serum orosin. Not only are the proteins of the posthemolytic residues different in composition from the other known blood proteins but they are also characteristic substances themselves. The amino acid contents of the proteins of posthemolytic residue prepared from different samples of red blood cells of any one species were approximately constant, showing that the method of preparation yields a product of quite constant composition when applied to different samples of erythrocytes from the same species. Furthermore, the posthemolytic residues of different mammalian bloods contain an almost identical distribution of seven amino acids. The similarity among the posthemolytic residues of different species, demonstrated by the lipid studies (2), is equally shown to be true by the present work on the protein moiety.

TABLE II

Per Cent Average Basic Amino Acid Composition of Lipid-Extracted Posthemolytic Residue, Compared with Values in the Literature for Serum Orosin, Hemoglobin, and Cattle Fibrin

	Histidine	Arginine	Lysine
Posthemolytic residue	2.1	5.0	3.6
Serum orosin (16)	2.1	4.7	6.9
Hemoglobin (17)	7.5	3.3	7.9
Cattle fibrin (18)	2.5	7.7	10.1

The distribution of the total nitrogen of the posthemolytic residue in terms of the nitrogen of seven of its constituent amino acids has been calculated and the data presented in Table III. The values are calculated on the basis of total nitrogen determined on the separate preparations used for the analysis of the individual amino acids. These results show that the seven amino acids which have been determined account for 25.4 to 27.0 per cent of the total nitrogen of the protein. From the data in Table III the molecular ratios among the amino acids in the stromata have been calculated and these appear in Table IV. The average molecular proportions of the seven amino acids determined in the posthemolytic residue are histidine to arginine to lysine to tyrosine to tryptophane to cystine to methionine as 7:14.5:13:8:3:2:3.5.

TABLE III

*Nitrogen of Amino Acids Expressed As Per Cent of Total Nitrogen of Posthemolytic Residue**

Species	Histidine	Arginine	Lysine	Tyrosine	Tryptophane	Cystine	Methionine	Per cent N accounted for
Beef	4.0	12.0	4.8	1.6	1.2	0.7	1.2	25.5
Sheep	4.7	11.4	4.5	1.4	1.0	0.8	1.6	25.4
Horse	4.2	11.6	5.4	1.6	1.1	0.8	1.6	26.3
Hog	4.5	11.4	5.1	1.6	1.3	1.0	1.5	26.4
Human	4.3	11.8	5.8	1.7	1.2	0.8	1.4	27.0

* Values obtained by use of the percentage amino acid composition (Table I) and the nitrogen content of the sample analyzed for the particular amino acid. Since the per cent nitrogen given in Table I is an average of all those obtained on preparations of that species, a direct calculation of nitrogen distribution cannot be made from those data. There is approximate agreement in most instances with Table I.

TABLE IV

*Molecular Ratios of Amino Acids of Posthemolytic Residues**

Species	Histidine	Arginine	Lysine	Tyrosine	Tryptophane	Cystine	Methionine
Beef	6.5	15	12	8	3	2	3
Sheep	8	14	11.5	7	2.5	2	4
Horse	7	14.5	13.5	8	3	2	4
Hog	7.5	14	13	8	3	2.5	3.5
Human	7	15	14.5	8	3	2	3.5
Average	7	14.5	13	8	3	2	3.5

* The ratios are calculated to the nearest half number.

The amino acid analyses show that the general pattern of the amino acid composition of the posthemolytic residues of the five mammals is similar. There is at present no assurance that the protein moiety of the erythrocyte residue is composed of a single protein but it is certain that if there are two or more individual proteins which constitute this residue, they appear in all of the species in the same proportions.

SUMMARY

It has been found that the posthemolytic residue proteins of the red blood cells of five mammalian species are practically identical in composition with respect to histidine, arginine, lysine, tyrosine, tryptophane, cystine, and methionine. This similarity in amino acid distribution in the protein moiety finds its counterpart in the constancy of the various lipid fractions as per cent of the total lipid portion of the erythrocyte residue. The average ratios among the amino acids in the protein fraction of stroma are histidine 7, arginine 14.5, lysine 13, tyrosine 8, tryptophane 3, cystine 2, methionine 3.5.

BIBLIOGRAPHY

1. Bernstein, S. S., Jones, R. L., Erickson, B. N., Williams, H. H., Avrin, I., and Macy, I. G., *J. Biol. Chem.*, **122**, 507 (1938).
2. Erickson, B. N., Williams, H. H., Bernstein, S. S., Avrin, I., Jones, R. L., and Macy, I. G., *J. Biol. Chem.*, **122**, 515 (1938).
3. Van Slyke, D. D., *J. Biol. Chem.*, **71**, 235 (1926-27).
4. Block, R. J., *J. Biol. Chem.*, **106**, 457 (1934).
5. Block, R. J., *Proc. Soc. Exp. Biol. and Med.*, **37**, 580 (1938).
6. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 89 (1929).
7. von Deseö, D., *Biochem. Z.*, **271**, 142 (1934).
8. Graff, S., Maculla, E., and Graff, A. M., *J. Biol. Chem.*, **121**, 81 (1937).
9. Baernstein, H. D., *J. Biol. Chem.*, **115**, 25 (1936).
10. Jorpes, E., *Biochem. J.*, **26**, 1488 (1932).
11. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
12. Vickery, H. B., and White, A., *J. Biol. Chem.*, **103**, 413 (1933).
13. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
14. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, suppl. 78 (1929).
15. Vickery, H. B., and White, A., *J. Biol. Chem.*, **99**, 701 (1932-33).
16. Block, R. J., *J. Biol. Chem.*, **105**, 455 (1934).
17. Block, R. J., *J. Biol. Chem.*, **105**, 663 (1934).
18. Bergmann, M., and Niemann, C., *J. Biol. Chem.*, **115**, 77 (1936).
19. Vickery, H. B., and White, A., *Proc. Soc. Exp. Biol. and Med.*, **31**, 6 (1933).

THE DENATURATION OF SERUM ALBUMIN

DIFFUSION AND VISCOSITY MEASUREMENTS OF SERUM ALBUMIN IN THE PRESENCE OF UREA*

By HANS NEURATH† and ARTHUR M. SAUM

(*From the Department of Chemistry, Cornell University, Ithaca, and the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*)

(Received for publication, December 7, 1938)

A large number of investigations have been carried out within recent years, concerning the influence of urea and related substances on proteins, and they have furnished valuable information regarding the phenomena of protein dissociation and protein denaturation.

Determinations of the molecular weight of proteins, by osmotic pressure methods and by sedimentation analysis, revealed that urea may produce *dissociation* of proteins (1–5). Amandin, excelsin, thyroglobulin, myogen, hemoglobin, and others become readily dissociated when dissolved in 50 per cent urea solutions, whereas proteins such as serum albumin, egg albumin, pepsin, and gliadin seem to resist splitting completely (1, 4, 6, 7).

Denaturation of proteins by urea and other amides, on the other hand, manifests itself in an increase of the number of detectable disulfide and sulfhydryl groups (8–10), and, in many instances, in coagulation of the denatured protein after removal of the denaturing agent.

Evidence has also accumulated that protein denaturation is accompanied by a change in protein structure from a condensed to a more extended configuration (11–14). Such structural changes should greatly affect the rate of diffusion of protein molecules and the relative viscosity of protein solutions, as elongated molecules exhibit a lower diffusion constant than do spherical molecules of equal volume, whereas the viscosity of solutions increases as the molecules become more asymmetrical. With these considerations in mind, we have carried out diffusion and viscosity measurements of serum albumin in the presence of urea. This protein has been chosen, since its molecular weight seems to remain unchanged during urea denaturation, thus allowing the observed effects on diffusion and viscosity to be ascribed primarily to changes in molecular shape. The results obtained were mentioned briefly on a previous occasion (15) and are described here in full.

---

* Part of a thesis presented by A. M. Saum to the Graduate School of Cornell University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, September, 1938.

† George Fisher Baker Research Fellow, Cornell University, 1936–38. That part of the work carried out at Duke University was supported by a grant from the Lederle Laboratories, Inc., Pearl River, New York.

Diffusion and Viscosity

The molecular weight of spherical molecules may be calculated directly from diffusion data, with the Southerland-Einstein equation (16)

$$D = kT/6\pi\eta r \tag{1}$$

in which k is the Boltzmann constant and r the molecular radius. Corrections for the shape factor have to be applied, however, if the molecules deviate from spherical shape. Perrin's equation (17) for the diffusion of elongated particles provides a relation between the observed diffusion constant, D, the diffusion constant, D_0, which would obtain if the molecules were spherical, and the ratio of the long to short axis of a prolate ellipsoid of revolution, b/a.

$$\frac{D}{D_0} = \frac{\sqrt[3]{a^2/b^2}}{\sqrt{1 - (a^2/b^2)}} \ln \frac{1 + \sqrt{1 - (a^2/b^2)}}{a/b} \tag{2}$$

The ratio of the observed diffusion constant, D, to the diffusion constant, D_0, may also be expressed as a function of the molecular weight, when the following equation (18) is used,

$$\frac{f}{f_0} = \frac{D_0}{D} = \frac{\dfrac{M(1 - V\rho)}{s}}{6\pi\eta N(3MV/4\pi N)^{1/3}} \tag{3}$$

in which f/f_0 is the dissymmetry constant, ρ the density of the solution, η the viscosity of the solvent, V the partial specific volume of the solute, s the sedimentation constant, and N the Avogadro number. For a given molecular weight, M, D_0 should be constant, whereas any change of D may be ascribed to changes in shape.

From Equations 2 and 3, the dissymmetry constant of native serum albumin (molecular weight 67,100) is 1.2 (18), and the ratio of the axes is 4.3 (15).

Values for b/a may likewise be estimated from viscosity data. In fact, such a method of computation should allow the validity of viscosity equations to be tested, since by substitution of b/a, obtained from viscosity measurements, in Equations 2 and 1, the molecular weight may be calculated (19, 15) and compared with the known value. We have used in this investigation Kuhn's equation (20) which relates the relative viscosity, η/η_0, and the volume concentration, φ, to the ratio of the axes, b/a.

$$(\eta/\eta_0) - 1 = \varphi(2.5 + (b/4a)^2) \qquad (4)$$

Although Kuhn's equation is subject to limitations and objections (21), its application proved to be suggestive for the estimation of shape factors of closely related substances such as proteins (15).

EXPERIMENTAL

Materials

Serum Albumin—Serum albumin was prepared by the method of Young (22) and of McFarlane (23). 5.5 liters of fresh horse blood were centrifuged and the corpuscles removed. The clear, yellow serum was then shaken with a total of 2 liters of ether, in small portions, to remove fats and lipids. An equal volume of saturated ammonium sulfate solution was added, slowly with constant stirring, and the mixture allowed to stand overnight. After filtration, an amount of 10 per cent acetic acid just sufficient to produce slight turbidity was added to the filtrate. This brought the pH of the solution to 6, and by the slow addition of more acid the pH was further lowered to about 5. The precipitated crystals were collected, dissolved in about 2.5 liters of distilled water, and recrystallized by the addition of an equal volume of saturated ammonium sulfate solution. When the material was filtered and the crystals redissolved in distilled water, almost no insoluble residue remained. Crystallization was repeated twice and the final product dissolved in distilled water, to a volume of about 2 liters. This solution was dialyzed against distilled water through cellophane membranes for several days and subsequently electrodialyzed in Pauli's apparatus with voltage gradually increasing from 10 to 220 volts. The protein concentration, as determined by drying at 104° for 18 hours, was 35.0 mg. per cc. The protein was crystallizable after electrodialysis.

Egg Albumin—Egg albumin was prepared according to the method of Kekwick and Cannan (24). The protein, obtained from fresh hen's eggs, was recrystallized three times, dialyzed, and electrodialyzed. The purified material was completely recrystallizable and had a pH of 4.78, a specific conductivity of 1.18×10^{-5} mho, and a concentration of 19.75 mg. per cc. Owing to the ease of surface denaturation of egg albumin, the solution was reelectrodialyzed from time to time and its concentration redetermined.

Measurements

The protein solutions were made up in small, 16 cc. volumetric flasks to which measured amounts of the stock protein and buffer solutions, along with any other materials whose effects upon the diffusion process was being investigated, were added and diluted with distilled water to the proper volume. 50 cc. of the corresponding solvent were then prepared, identical in all solute components except protein. The protein solution was placed in a small cellophane bag and allowed to dialyze for at least 24 hours against the corresponding solvent. After dialysis the solutions were stored separately, in small glass-stoppered bottles, until placed in the diffusion cell.

Apparatus

The diffusion apparatus was built in this laboratory according to the descriptions given by Lamm (25, 26). A mercury arc lamp served as light source and Corning light filters No. 551 and No. 512 in addition to a solution of cupric chloride in methanol were used to eliminate all but the green, 546 mμ line of the mercury spectrum. Monochromatic light proved to be useful for correcting the chromatic aberration of the simple biconvex quartz lens of the camera. The camera lens had a focal length of 59.75 cm. and was adjusted to an aperture of *f*: 60. All materials in the light path of the apparatus were of either quartz or Corex D glass, to permit use of light of any wave-length, including the ultraviolet. The thermostat was kept at 25° ± 0.002°. The scale was a transparent mm. scale subdivided into 0.5 mm. in the region near the boundary. Eastman Kodak spectroscopic plates GV, sensitized for the green line, were used. The time of exposure was 1½ minutes, the photographic enlargement factor 1.201. Scale line readings were made with a Zeiss microcomparator capable of being read to 1 μ. The readings were reproducible to within 2 μ.

In the early stage of this investigation the cylindrical cell described by Lamm and Polson (25) was used. Most of the experiments, however, were performed with a stainless steel cell with plane parallel windows (26). A slide, made of lucite, with a screw arrangement, permitted the boundary between solvent and solution to be formed in a simple and smooth manner. The distance of the cell from the scale was adjusted according to the protein concentration, the distance being smaller for higher protein concentrations and *vice versa*.

Viscosity measurements were carried out at 25° ± 0.03° in Ostwald pipettes, with flow times of about 60 seconds for distilled water. Corrections for density were applied in all cases. They were determined at room temperature with pycnometers of 2 or 5 cc. content, respectively.

Methods of Calculation

The scale line displacement was plotted against the position of the displaced lines in the usual manner and diffusion constant calculated by three of the methods described by Lamm (25). These are the "maximum height" method, the method of "successive analysis," and the "statistical" method (Equations 2, 4, and 9 of Lamm and Polson's paper (25)). At least two of these methods were employed in every experiment in order to examine the state of dispersion of the solutions. In the calculation of the diffusion constant, corrections were applied for the photographic enlargement factor as well as for the optical distances of the lens from the cell and from the scale respectively. In the following, D designates the observed diffusion constant and D' the diffusion constant if corrections for the viscosity of the solvent were applied, according to the equation

$$D' = D(\eta/\eta_0) \tag{5}$$

η/η_0 is the relative viscosity of the solvent in respect to water. Diffusion constants are expressed in sq. cm. per second.

Results

Diffusion Measurements

The diffusion apparatus was tested for accuracy by measuring the diffusion constant of egg albumin in the presence of 0.2 M acetate buffer of pH 4.8 and of sodium chloride in a concentration of 0.2 M. The diffusion constant, corrected for the viscosity of the solvent, was found to be 8.61 × 10^{-7} sq. cm. per second at 25°, in good agreement with the data of the literature, recalculated for the same temperature (25, 27, 28).

Table I

Diffusion Constants of Serum Albumin in 0.2 M Acetate Buffer, pH 5.2, and 0.2 M NaCl

Protein concentration	Time	$D \times 10^7$
per cent	*sec.*	
2.00	64,680	6.44
	86,940	6.35
	151,080	6.43
1.33	67,620	6.10
	76,440	6.12
	86,940	6.28
	94,860	6.01
1.06	23,940	6.52
	39,060	6.43
	90,220	6.26
Average		6.29
D'		6.80

Table I summarizes our findings with three different concentrations of serum albumin in 0.2 M acetate buffer, pH 5.2, and 0.2 M NaCl.

Lamm and Polson (25) reported a value of $D'_{20} = 6.47 \times 10^{-7}$ as the average value for concentrations ranging from 0.93 to

2.0 per cent serum albumin. Recalculated, this value yields $D'_{25} = 7.36 \times 10^{-7}$ which is considerably higher than our value. Polson later reported a value of $D'_{20} = 6.10 \times 10^{-7}$ (19) which is equivalent to $D'_{25} = 6.98 \times 10^{-7}$, in good agreement with our data.

Diffusion measurements of serum albumin in aqueous urea solutions were carried out 24 hours or more after preparation of the solutions. No influence of time of storage on the diffusion constants could be observed. Measurements were carried out with two different protein concentrations at a given concentration of urea. These were 1.75 and 1.16 per cent serum albumin respectively.

TABLE II

Diffusion Constants of Serum Albumin in Solutions of 1.5 and 6 M Urea, 0.2 M Acetate Buffer, pH 5.2, and 0.2 M NaCl

t = time in seconds, H_m = maximum ordinate, 2μ = distance between the inflection points, H = ordinate of the abscissa, x, N = summation over all ordinates, D_m = diffusion constant calculated by the maximum height method, D_s = diffusion constant calculated by the method of successive analysis, D = diffusion constant calculated by the statistical method, and D' = diffusion constant corrected for the viscosity of the solvent.

t	$H_m \times 10^4$	2μ	$N \times 10^3$	$H \times 10^4$	x	$D_m \times 10^7$	$D \times 10^7$	$D_s \times 10^7$
1.5 M urea, 1.75% protein								
60,180	396	0.655				5.28		
88,380	327	0.810	336.4			5.50	5.41	
151,500	257	1.015	345.6			5.39	5.31	
Average						5.39	5.36	
D'						6.10	6.06	
6 M urea, 1.66% protein								
23,400	778	0.290				2.66		
67,620	466	0.500				2.75		
90,900	400	0.595	582.6	332	−0.18	2.88	2.66	2.84
				242	−0.30			2.92
				159	−0.40			2.83
				314	0.20			2.73
				194	0.35			2.76
Average						2.76	2.66	2.82
D'						4.19	3.96	4.28

Table II illustrates the agreement between the diffusion constants computed by the various methods of calculation for serum albumin in solutions of 1.5 and 6 M urea respectively, in addition to the buffer components present. In most cases normalized curves were constructed from the data, and, with the exception of the 0.5 M urea solution, they coincided exactly with the ideal curves, showing that the protein was monodisperse.[1] Taking an average of all observed diffusion constants at the various urea concentrations, we obtained the values summarized in Table III. In an attempt to decide whether the change of the diffusion constant may be due to the inapplicability of the viscosity correction (Equation 5), we have carried out diffusion measurements of serum albumin in the presence of sodium salicylate which, even in low concentration, produces a manifold increase of the viscosity of the solvent. The observed diffusion constants of serum albumin in the presence of 0.2 M sodium chloride and 0.2 M acetate buffer, pH 5.2, were for 1 M and 3.5 M sodium salicylate 4.29×10^{-7} and 9.0×10^{-8} respectively. Correcting for the viscosity of

TABLE III

Average of All Observed (D) and Corrected (D') Diffusion Constants of Serum Albumin in Relation to Concentration of Urea in Solution

All experiments refer to solutions containing 0.2 M acetate buffer, pH 5.2, and 0.2 M NaCl in addition to protein and urea. η/η_0 is the relative viscosity of the solvent in respect to water.

Concentration of urea	$D \times 10^7$	$\frac{\eta}{\eta_0}$	$D' \times 10^7$
M			
0	6.40	1.070	6.85
0.5*	5.68	1.092	6.20
1.5	5.37	1.133	6.08
3.0	4.62	1.231	5.69
4.5	3.39	1.309	4.45
6.0	2.82	1.512	4.27
6.66	2.55	1.590	4.15

* This solution proved to be slightly polydisperse.

---

[1] Slight deviations from monodispersity, due to the presence of two or more molecular species of different molecular weights or shapes but almost identical diffusion constant, could only be detected by sedimentation analysis.

the solvent, we obtained the values of 6.6×10^{-7} and 6.3×10^{-7}. This is in close agreement with the values found for the protein in aqueous buffer solution, in spite of a viscosity correction of 700 per cent in the higher salicylate concentration.

Tiselius and Gross (28) found the diffusion constant of proteins to be independent of the pH within a certain range, and we found the same to be true for serum albumin in the presence of 3 M urea. This is illustrated by Table IV.

In investigating the nature of the process whereby the protein undergoes changes, as indicated by diffusion measurements in urea solutions, we performed experiments to determine the concentration of urea that was sufficient to prevent the coagulation of dilute serum albumin solutions by heat. Twelve 4 cc. samples containing 1.18 per cent protein, acetate buffer 0.2 M at pH 5.2, 0.2 M NaCl, and urea ranging from 1.25 to 4.0 M, in steps of 0.25 M, were made up in small test-tubes. They were allowed to stand for at least 24 hours and then immersed in boiling water. A precipitate was formed almost immediately in all cases, but on continued heating for 35 minutes the precipitate in the solutions containing more than 3.0 M urea disappeared completely. On repetition of the experiments with solutions containing the same protein and urea concentrations, but no buffer and sodium chloride, the same results were obtained except that the lower limit of the necessary urea concentration for preventing heat coagulation was reduced to 2.0 M. The results of these latter experiments are doubtful, however, as the solutions became alkaline upon heating, with pH in all cases higher than 7.5. This in itself would help to prevent coagulation.

TABLE IV

Diffusion Constants of Serum Albumin in Presence of 3 M Urea, in Relation to pH

The protein concentration was 1.16 per cent.

Buffer solution	pH	$D' \times 10^7$
Acetate buffer 0.2 M, NaCl 0.2 M	5.23	5.69
Phosphate " 0.2 " " 0.2 "	6.30	5.50
Acetate " 0.2 " " 0.2 "	3.50	5.42

The effect of heat denaturation upon the properties of serum albumin was further investigated by determining the diffusion constant in acetate buffer of pH 3.23, and in acetate buffer of pH 5.25 plus 3.0 M urea, after these solutions had been heated for 15 minutes to 100°. The results of these investigations are given in Table V. The inconsistency of the D_m and D values in Table V, A is indicative of the very polydisperse character of heat-denatured serum albumin on the acid side of its isoelectric point. This was even more apparent when the normalized experimental points were compared with the ideal distribution curve. Although the various D_m and D values of Table V, B agree among themselves exceptionally well, the D_m values do not agree with the D values, and a comparison of the experimental and ideal diffusion

TABLE V

Diffusion Constants of Heat-Denatured Serum Albumin

t = time in seconds, H_m = maximum ordinate, 2μ = distance between the inflection points, N = summation over all ordinates, D_m = diffusion constant calculated by the maximum height method, D = diffusion constant calculated by the statistical method, D' = diffusion constant corrected for the viscosity of the solvent, and η/η_0 = relative viscosity of the solvent in respect to water. The protein concentration was 1.16 per cent.

t	$H_m \times 10^4$	2μ	$N \times 10^3$	$D_m \times 10^7$	$D \times 10^7$	$\frac{\eta}{\eta_0}$
A. Coagulation prevented by acetate buffer, pH 3.23, and 0.2 M NaCl						
59,760	630	0.205	492.9	0.854	2.59	1.032
72,960	502	0.290	506.7	0.521	2.08	
Average				0.688	2.34	
D'				0.710	2.41	
B. Coagulation prevented by 3.0 M urea in addition to 0.2 M acetate buffer, pH 5.2, and 0.2 M NaCl						
45,000	570	0.272		1.22		1.233
58,560	502	0.316	384.4	1.26	2.31	
73,800	446	0.348	336.4	1.22	2.27	
Average				1.23	2.29	
D'				1.52	2.82	

curve indicated that the material was only slightly less polydisperse than that obtained by heat denaturation at pH 3.23 in the absence of urea.

Viscosity Measurements

In these experiments, the relative viscosity of the protein solution was compared with that of the respective solvent by keeping the composition of the solvent constant during a given set of determinations and varying the protein concentration. The protein concentration was never higher than 1.75 per cent in order to avoid structural viscosity effects, as they have been observed at high protein concentrations (29). The volume concentration was calculated from the weight concentration and the partial specific volume of serum albumin (30). Comparative measurements indicated that the partial specific volume was not changed by the addition of urea, which is in keeping with Steinhardt's (4) observations for hemoglobin. Furthermore, we have ascertained that the viscosity of the solutions of protein in the presence of urea was independent of time, if sufficient time after mixing was allowed for the reaction to become complete. The solutions contained in addition to protein and urea, 0.2 M acetate buffer at pH 5.2, and 0.2 M sodium chloride. By plotting the specific viscosity, $((\eta/\eta_0) - 1)$, against the volume concentration of protein, φ, the limiting slope was determined graphically and is listed with the other data in Table VI. Deviations of the limiting slope from the value of 2.5, demanded by the Einstein equation, was taken as evidence for the molecules being non-spherical. The ratio of the axes, on the assumption of prolate ellipsoids of revolution, was calculated from the limiting slope with the aid of Equation 4. The results of these calculations are listed in Column 5 of Table VI. Columns 6 and 7 refer to the dissymmetry constants and molecular weights respectively, calculated with Equations 2 and 1.

DISCUSSION

From the observed diffusion constants of serum albumin in the presence of urea, D', and the calculated value for the diffusion constant of a spherical molecule of the molecular weight of 67,100 (30), $D_0^{25} = 8.22 \times 10^{-7}$, we have calculated the respective dis-

TABLE VI

Viscosity Measurements of Serum Albumin in Buffered Urea Solutions

η/η_0 = relative viscosity of solution in respect to solvent; φ = volume concentration of protein; b/a = ratio of long to short axis, on the basis of Kuhn's equation; D_0/D = dissymmetry constant; M = molecular weight.

Urea (1)	Protein $\varphi \times 10^3$ (2)	$\frac{\eta}{\eta_0}$ (3)	Limiting slope (4)	$\frac{b}{a}$ (5)	$\frac{D_0}{D}$ (6)	M (7)
M per l.						
0.0	0.00	1.000	6.50	8.0	1.43	51,800
	3.29	1.021				
	6.58	1.044				
	9.87	1.071				
	13.09	1.093				
0.5	0.00	1.000	6.60	8.3	1.45	67,300
	3.29	1.022				
	6.58	1.045				
	9.87	1.071				
	13.09	1.098				
1.5	0.00	1.000	7.40	8.9	1.49	69,200
	3.29	1.024				
	6.58	1.049				
	9.87	1.075				
	13.09	1.104				
3.0	0.00	1.000	8.7	10.0	1.55	72,200
	3.29	1.028				
	6.58	1.056				
	9.87	1.085				
	13.09	1.115				
4.5	0.00	1.000	16.4	14.9	1.77	100,000
	2.17	1.037				
	4.33	1.070				
	6.05	1.099				
	8.66	1.141				
6.0	0.00	1.000	19.6	16.5	1.85	99,200
	3.14	1.061				
	6.28	1.131				
	9.43	1.214				
	12.60	1.294				
6.66	0.00	1.000	22.6	17.9	1.91	98,100
	2.99	1.067				
	5.98	1.139				
	8.98	1.222				
	11.97	1.308				

symmetry constants, and, with the aid of Equation 2, the ratios of the axes, b/a. These values are listed in Columns 3 and 4 of Table VII. It may be seen that, according to these data, the protein molecules become more elongated as the urea concentration increases. The discontinuity in the increase of the shape factors in the neighborhood of 3 M urea manifests itself also, in that this is the minimum concentration of urea which prevents the flocculation of serum albumin by heat. There are, apparently, abrupt structural changes associated with this particular composition of the solvent.

TABLE VII

Diffusion Constants and Shape Factors of Serum Albumin in Urea Solutions

Molecular weight 67,100, $D_0^{25} = 8.22 \times 10^{-7}$. a = length of short axis of molecules.

Urea (1)	$D' \times 10^7$ (2)	$\frac{D_0}{D'}$ (3)	$\frac{b}{a}$ (4)	a (5)
M				Å.
0	6.85	1.20	4.3	33.9
0.5	6.20	1.33	6.1	29.5
1.5	6.08	1.35	6.5	28.9
3.0	5.69	1.44	8.0	25.6
4.5	4.45	1.85	16.5	21.2
6.0	4.27	1.93	18.3	20.5
6.66	4.15	1.98	19.4	20.1

The absolute dimensions of the serum albumin molecule, calculated from b/a and the molecular weight, approach with increasing urea concentration values of 20.1 and 358.8 Å. for the short and long axis respectively (Table VII, Column 5). It will be remembered that the side chain spacing of polypeptide chains, of the folded as well as of the fully extended type, is about 9.5 to 10 Å. (31). The speculation may be allowed that, in the urea-denatured state, the serum albumin molecule consists of two polypeptide chains held together by disulfide groups of cystine residues (13). This hypothesis would concur with the findings (8) that with proteins which do not contain sulfhydryl groups, but which are rich in cystine, such as serum albumin, an increase of the number of detectable disulfide groups takes place upon denaturation. It may indeed be expected that in an extended configuration of the protein molecule, disulfide groups are more accessible for the testing reagent than in a condensed structure.

Evidence for the occurrence of unfolding of polypeptide chains upon protein denaturation is also available from surface film work and from x-ray studies. Bull (14) has shown that monolayers of native (surface-denatured), heat-denatured, and urea-denatured egg albumin respectively, possess similar structures and properties, and their identity with the structure of β-keratin has recently been established (32, 33). x-Ray studies of heat-denatured proteins, and of protein fibers produced by treatment of egg albumin and edestin with concentrated urea solutions, point in the same direction (13).

Our viscosity data, interpreted with Kuhn's equation, are in qualitative accord with the diffusion data, as may be seen from Table VI, Columns 5 and 6. The absolute values for b/a are, however, too high in low concentrations and too low in high concentrations of urea, as compared with the values obtained from diffusion experiments. Accordingly, molecular weights calculated from viscosity data with Equations 4, 2, and 1 are too low and too high respectively[2] (Table VI, Column 7). Kuhn's equation is for the limiting case of strong Brownian motion or negligible velocity gradient. Restrictions of geometrical and physical nature arise by the application of Kuhn's equation to molecules which differ in shape from the model which has been used in Kuhn's calculations. The application of Einstein's incompressibility term for spherical particles (2.5) to non-spherical particles may likewise be open to objections.[3] We have shown previously (35) that the assumption of strong Brownian motion may not be fulfilled if very elongated protein molecules are allowed to flow through the capillary of an Ostwald viscosimeter. It is probable that owing to the orientation of such anisotropic particles in the streaming liquid the observed viscosity is lower than that which would be observed if the orientation of the particles were random. Hence, the actual viscosity of serum albumin in high urea concentration may be higher than that reported in this paper. This problem is the subject of further investigations and will be discussed in a subsequent publication.

---

[2] Interpretation of the viscosity data by other equations, such as that suggested by Eisenschitz (34), would lead to even greater deviations from the theoretical value of the molecular weight.

[3] We are indebted to Dr. M. L. Huggins for helpful discussions of this problem.

SUMMARY

1. Diffusion and viscosity measurements of native, urea-denatured, and heat-denatured serum albumin have been carried out. The diffusion constant has been found to decrease with increasing concentration of urea, whereas the relative viscosity increases with increasing urea concentration. Heat denaturation, carried out in the presence of 3 M urea or at pH 3.2 respectively, likewise results in a decrease of the diffusion constant. Solutions of urea-denatured serum albumin have been found to be monodisperse if the urea concentration is higher than 0.5 M, whereas those of heat-denatured serum albumin are heterodisperse.

2. Calculations of molecular shapes from diffusion data, on the assumption of unchanged molecular weight, indicate that the protein molecules become more elongated as the urea concentration increases. The absolute dimensions, for the model of an ellipsoid of revolution, approach values of 20 and 359 Å. for the short and long axes of the protein molecule respectively. It is suggested that in the fully denatured state the serum albumin molecule consists of two extended polypeptide chains probably held together by disulfide groups of cystine residues.

3. Viscosity data, interpreted by Kuhn's equation, are in qualitative accord with the diffusion data. It has been found, however, that molecular weights calculated from diffusion and viscosity data are too low in regions of small dissymmetry constant and too high in those of large dissymmetry constant. These deviations are related to the limitations of Kuhn's viscosity equation.

BIBLIOGRAPHY

1. Burk, N. F., and Greenberg, D. M., *J. Biol. Chem.*, **87**, 197 (1930).
2. Burk, N. F., *J. Biol. Chem.*, **120**, 63 (1937).
3. Weber, H. H., and Stover, R., *Biochem. Z.*, **259**, 269 (1933).
4. Steinhardt, J., *J. Biol. Chem.*, **123**, 543 (1938).
5. Lundgren, H. P., *Nature*, **138**, 122 (1936).

6. Burk, N. F., *J. Biol. Chem.*, **98**, 353 (1932).
7. Burk, N. F., *J. Biol. Chem.*, **124**, 49 (1938).
8. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **18**, 307 (1935); **19**, 427, 439 (1936).
9. Hopkins, F. G., *Nature*, **126**, 328, 383 (1930).
10. Greenstein, J. P., *J. Biol. Chem.*, **125**, 501 (1938).
11. Astbury, W. T., and Lomax, R., *J. Chem. Soc.*, 846 (1935). Astbury, W. T., *Nature*, **137**, 803 (1936).
12. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, **22**, 439 (1936).
13. Astbury, W. T., Dickinson, S., and Bailey, K., *Biochem. J.*, **29**, 2351 (1935).
14. Bull, H. B., *J. Biol. Chem.*, **125**, 585 (1938).
15. Neurath, H., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6**, 196 (1938).
16. Einstein, A., *Z. Elektrochem.*, **14**, 235 (1908).
17. Perrin, F., *J. physiq. radium*, **7**, 1 (1936).
18. Svedberg, T., *Chem. Rev.*, **14**, 1 (1934); **20**, 81 (1937).
19. Polson, A., *Nature*, **137**, 740 (1936).
20. Kuhn, W., *Z. physik. Chem., Abt. A*, **161**, 1 (1932).
21. Huggins, M. L., *J. Physic. Chem.*, **42**, 911 (1938).
22. Young, E., *Proc. Roy. Soc. London, Series B*, **93**, 15 (1922).
23. McFarlane, A., *Biochem. J.*, **29**, 416 (1935).
24. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 235 (1936).
25. Lamm, O., and Polson, A., *Biochem. J.*, **30**, 528 (1936).
26. Lamm, O., *Nova acta regiæ soc. scient. Upsaliensis*, **10**, No. 6 (1937).
27. Herzog, R. O., *Z. Elektrochem.*, **13**, 533 (1907). Groh, J., *Biochem. Z.*, **173**, 249 (1926).
28. Tiselius, A., and Gross, D., *Kolloid-Z.*, **66**, 11 (1934).
29. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **15**, 341 (1932).
30. von Mutzenbecher, P., *Biochem. Z.*, **266**, 250 (1933).
31. Astbury, W. T., and Woods, H. J., *Nature*, **126**, 913 (1930); *Phil. Tr. Roy. Soc. London, Series A*, **232**, 333 (1933).
32. Astbury, W. T., Bell, F. O., Gorter, E., and Van Ormondt, J., *Nature*, **142**, 33 (1938).
33. Neurath, H., and Bull, H. B., *Chem. Rev.*, **23**, 391 (1938).
34. Eisenschitz, R., *Z. physik. Chem., Abt. A*, **158**, 78 (1931); **163**, 133 (1933).
35. Neurath, H., and Saum, A. M., *J. Biol. Chem.*, **126**, 435 (1938).

EFFECT OF AMINO ACIDS ON ANEMIA CAUSED BY DEAMINIZED CASEIN*

By RALPH E. GUERRANT and ALBERT G. HOGAN

(From the Department of Agricultural Chemistry, University of Missouri, Columbia)

(Received for publication, January 30, 1939)

Deaminized casein is grossly inadequate as the sole source of nitrogen, so in our studies of its nutritional properties it has been combined with other proteins. It has been shown in earlier papers (1, 2) that when deaminized casein is combined with gelatin or gliadin, or a laboratory preparation of lactalbumin, and fed to rats they become severely anemic, fail to grow, and die. Surprisingly enough, if deaminized casein is combined with normal casein in the diet, the rats do not become anemic and they grow normally. If normal casein is hydrolyzed and the hydrolysate combined with an anemia-producing ration, then the animals recover from the anemia and growth is normal. It is almost certain that the anti-anemic activity of hydrolyzed casein is explained by its amino acid content; the purpose of this paper is to report subsequent attempts to obtain the active agent in more concentrated form.

EXPERIMENTAL

In order to save time and expense the rats are first made anemic by the procedure of Elvehjem and Kemmerer (3) before the experimental period begins. They are then placed in individual cages and transferred to the experimental ration, No. 2149, made up as follows:

Deaminized casein	10.0	Agar	2.0
Lactalbumin	15.0	Salts (4)	4.0
Corn-starch	52.5	Cod liver oil	2.0
Milk fat	12.5	Water extract of yeast	2.0

---

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series, No. 596.

When the various amino acid mixtures to be tested were included in the ration, they displaced an equivalent weight of starch. In later descriptions of individual rations, therefore, only the kind and amount of the nitrogenous constituents will be specified, as the other constituents either remain constant or are readily calculated.

Fractionation of Hydrolyzed Casein—The methods are combinations of those applied previously by other investigators, and will be indicated by a brief description of the first instance in which the procedure now followed was used. 3 kilos of casein were hydrolyzed with 1490 gm. of concentrated sulfuric acid and 3 liters of water for each 500 gm. of casein. The sulfuric acid was then removed with barium hydroxide, and the amino acid mixture was concentrated to 20 liters. One-sixth of this was removed for another purpose but tests showed that it was effective in curing anemic rats. The remainder of the amino acid solution was concentrated to a volume of 4.5 liters. This was packed in ice for 2 days and then the soluble and insoluble amino acids were separated by filtration. The insoluble amino acids were washed with ice water and the washings added to the more soluble portion. When tested, it was found that the insoluble amino acids were only slightly effective in curing the anemic animals, and the soluble amino acids were highly effective.

Since the soluble amino acids contained most of the activity, they were subjected to further fractionation. Solid $Ba(OH)_2$ was added in 30 per cent excess (5) and the mixture was poured into 4 volumes of 95 per cent ethyl alcohol and packed in ice for 2 days. The dicarboxylic acid fraction which was precipitated was filtered off and washed with 70 per cent alcohol. The alcohol was removed from the filtrate by distillation under reduced pressure, and the excess barium was precipitated with sulfuric acid and filtered off. The dicarboxylic acid fraction was decomposed with sulfuric acid, and, after removal of the barium sulfate, both fractions were examined to see which contained the larger proportion of the antianemic agent. The dicarboxylic acid fraction was ineffective in curing anemia and it accelerated the rate of growth slightly or not at all. The amino acids not precipitated as barium salts by alcohol were very effective both in curing anemia and in supporting growth.

For the next stage in concentration two methods have been considered, the first of which is the butyl alcohol extraction method of Dakin (6). Half of the remaining amino acids was used, and it developed that the butyl alcohol-soluble portion had very slight activity and that the insoluble portion was highly active. All details will be omitted though, as this procedure was abandoned when it became apparent that the second method gave some promise of being more useful at this stage. In the second method the remainder of the amino acid mixture was subjected to copper salt fractionation as described by Caldwell and Rose (7). The three copper salt fractions obtained were freed of copper by hydrogen sulfide, and the solutions of the amino acids were evaporated to dryness. The amino acids whose copper salts are soluble in water were gummy, so they were partially converted into calcium salts in order to make them friable enough to be handled easily. 5 gm. of calcium hydroxide were added to the fraction that is soluble in methyl alcohol, and 20 gm. to the fraction which is insoluble in methyl alcohol. The weights of the three fractions are (1) copper salts insoluble in water, 27.0 gm.; (2) copper salts soluble in water and soluble in methyl alcohol, 50.0 gm.; (3) copper salts soluble in water but insoluble in methyl alcohol, 200.0 gm.

Inspection of the weights shows that the amount of amino acids whose copper salts are insoluble in water is relatively small, so if this fraction contains most of the active principle it must be very concentrated. At first therefore only 50 mg. daily of this fraction were supplied as a supplement to the basal diet. When this amount was found ineffective, it was increased to 100 mg. and later to 200 mg. daily, but even the latter amount was without retarding effect on the anemia.

The amino acids from the copper salts soluble in both water and methyl alcohol were supplied in amounts of 50 and 100 mg. daily, as a supplement to the basal diet, and also as 2 to 4 per cent of the ration. At none of these levels was this fraction effective in curing anemia.

There remains then Fraction 3, the amino acids whose copper salts are insoluble in methyl alcohol but soluble in water. The rations which contained this fraction were modifications of Ration 2149, obtained by substituting the amino acid mixture for an equal weight of starch. The following are the substituted amounts: Ration 3038, 5 per cent; Ration 3039, 10 per cent; Ration 3042, 2 per cent; Ration 3075, 3 per cent.

When the rats received the ration which contained 5 per cent of

Fraction 3, they recovered as quickly and grew as rapidly as when it contained 10 per cent, or as when it contained 10 per cent of casein itself. 2 per cent of this fraction was not enough, as only one of the three animals on this amount recovered. Another preparation of this copper salt fraction, which had been extracted still more thoroughly with methyl alcohol, cured anemia and supported growth at a level of 3 per cent. 100 mg. daily of this second preparation, as a supplement to Ration 2149, is not enough, as only one of the two animals observed recovered and it grew slowly. The other died at the end of 16 days. Additional data on the red cell counts and rate of growth are summarized in Fig. 1.

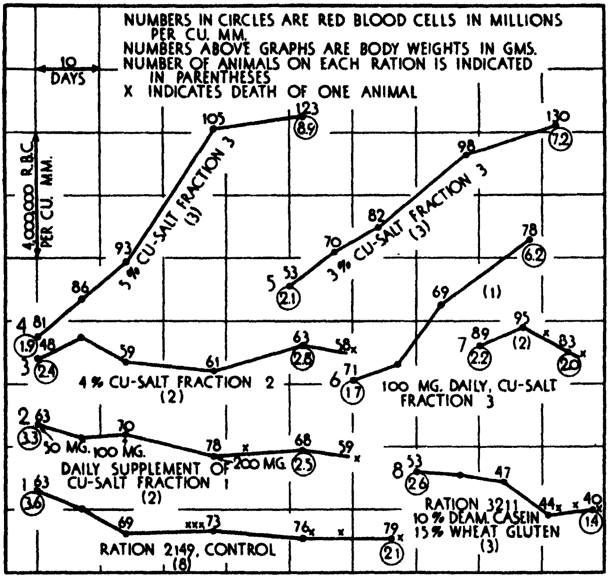


FIG. 1. The effect on anemia of various fractions of amino acids. Hydrolyzed casein was separated into three fractions by the copper salt method. The fraction which is soluble in water and insoluble in methyl alcohol, Graphs 4 to 7, cures anemia caused by deaminized casein. The other two fractions are inactive, Graphs 2 and 3. Wheat gluten is suitable for use in anemia-producing rations, Graph 8.

There is no doubt that Fraction 3 is the most active but it seemed possible that more than one factor might be concerned. If this supposition is correct, then a considerable part of one of the active agents might be in either the fraction which is soluble in methyl alcohol or in the fraction which is insoluble in water. To test this hypothesis the methyl alcohol-insoluble portion was supplemented with each of the others. The methyl alcohol-soluble fraction seemed to increase the growth rate but the recovery from anemia was not markedly affected. The amino acids from the water-insoluble fraction were altogether ineffective. It is concluded then that whatever the number of amino acids concerned may be, Fraction 3 is the most concentrated source of all of them.

The total potency of Fraction 3 is much less than that of the casein from which it was obtained. A portion of the active agent may have been destroyed by the chemical procedures, but more probably it was lost in the various stages of the separation process. A clear cut separation of amino acids cannot be obtained by the procedures employed.

Amino Acid Supplements—The next problem is the identification of the active agent, and though this has not been achieved the preliminary experiments will be described briefly. It was hoped that the distribution of the amino acids in the various copper salt fractions would be a useful guide, and so previous reports on the composition of these fractions were taken as a starting point. The amino acids whose copper salts are soluble in water, but insoluble in methyl alcohol (7), are glycine, glutamic acid, arginine, alanine, hydroxyglutamic acid, histidine, serine, tyrosine, and lysine.

Of these nine it is probable that only six are present in Fraction 3 in any considerable amount. Most of the tyrosine was precipitated in the first concentration of the entire amino acid mixture. At a later stage the barium salts of glutamic acid and hydroxyglutamic acid were precipitated with alcohol. This precipitate probably contained in addition a considerable portion of glycine (8).

Our chief interest was at first confined to the six amino acids that should be present in the active copper salt fraction. Hogan and Ritchie (1) had failed to find antianemic activity in lysine, and Smith and Stohlman (9) had been equally unsuccessful with lysine and histidine. Arginine, alanine, and serine were regarded as the most promising, therefore, but histidine was included also to confirm the report of Smith and Stohlman. Each of these was tested separately at a level of 50 mg. daily, as a supplement to the basal ration, but none effected any improvement in the anemic condition. It was then decided to try glycine, glutamic acid, and tyrosine, at levels of 100 mg. daily. Aspartic acid does not belong in Fraction 3 but since it was available it too was tried. All four were entirely inert.

Inasmuch as the results with the amino acids supposed to be in Fraction 3 had been disappointing, it was decided to try some of the others. It seemed probable that the substance sought is an essential amino acid, so threonine, valine, phenylalanine, leucine, and isoleucine[1] were tried separately, at levels of from 50 to 100 mg. daily. None was effective. Three essential amino acids remain then which have not been investigated at this time, lysine, methionine, and tryptophane. The reason for omitting lysine has been mentioned; it had not shown curative properties in earlier trials. According to Hogan and Ritchie (1) methionine and tryptophane had offered no promise of usefulness, so these two were also omitted.

Enlargement of Spleen—In our experience the only abnormality of the internal organs that has been consistent is an enlargement of the spleen, which often becomes several times the normal size. This was first observed on postmortem examination of rats that had died while severely anemic, and it was supposed at the time that the spleen was in some way involved in the disappearance of red blood cells. However, further study showed that the spleen may enlarge during recovery. It seems more probable then that the increase in size is related to an accelerated rate of formation of erythrocytes, rather than to an increased rate of destruction. Hamre and Miller (10) observed that there is a relation between the size of the spleen and recovery from nutritional anemia.

Fig. 2 shows that the spleens of anemic rats are from 1 to 4 times as heavy as those from normal animals (11) of comparable weight. It should be pointed out though that there is no definite ratio, as the increase in size depends on both severity of the anemia and the length of the anemic period. Some of the rats for which results are shown in Fig. 2 had been given a curative treatment and there is no reason to suppose that the spleens had decreased in size during recovery. The data give no indication as to whether the increase in size is persistent.

---

[1] Practically all of the amino acids used recently were obtained from commercial sources.

Suitability of Proteins for Studies of Anemia Caused by Deaminized Casein—To digress from the main theme for a moment, it should be mentioned that different preparations of lactalbumin are not equally suitable for studies of this type of anemia. Most of the preparations permit a slow rate of growth with no increase in the number of red blood cells, but on a few preparations the animals will recover spontaneously. It is assumed that this difference is due to variability during the separation process. The skim milk is heated with live steam in 50 gallon barrels in order to precipitate the albumin, and for various reasons there may be considerable variation in the time during which the protein is exposed to high temperatures. Recent papers (12, 13) indicate that this variability may be a factor of some importance. A mixture of gliadin and gelatin may be used but as these proteins are expensive a search was made for a substitute. It

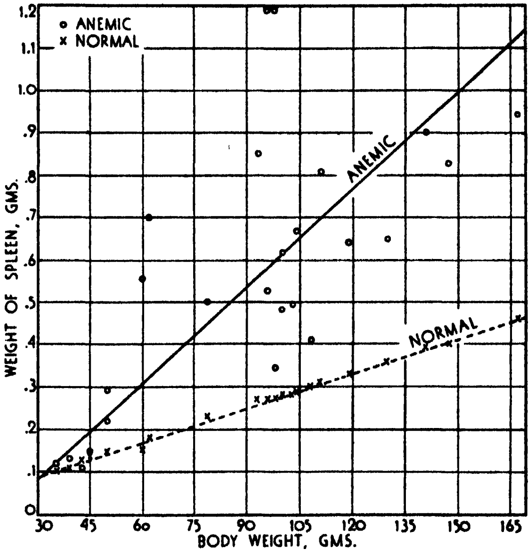


Fig. 2. Enlargement of spleen in anemic rats

developed that either wheat or corn gluten is satisfactory, but wheat gluten is preferable, as it has a higher biological value. When this protein is used, Ration 3211, it displaces the lactalbumin of Ration 2149. As shown in Fig. 1 (Graph 8), when rats receive deaminized casein and wheat gluten they not only become anemic, but they also fail to make any consistent gain in weight.

DISCUSSION

When it was first discovered that casein is effective, and lactalbumin ineffective, in preventing anemia due to deaminized casein, it was hoped that the percentages of amino acids they contain might explain this difference in behavior. According to the compilation published by Schmidt and Allen (14) six different amino acids are present in markedly smaller proportions in lactalbumin than in casein, as shown below.

	Casein	Lactalbumin		Casein	Lactalbumin
	per cent	*per cent*		*per cent*	*per cent*
Glutamic acid	21.8	12.9	Hydroxyproline	0.2	Trace
Phenylalanine	3.9	1.3	Tyrosine	4.5	2.0
Proline	7.6	3.8	Valine	7.9	3.3

As was mentioned previously, the possibility that glutamic acid, phenylalanine, tyrosine, and valine might be involved was tested by adding them directly to the anemia-producing ration, with negative results.

If any of the amino acids are involved in this syndrome, it seems highly probable that they belong to the group classified as essential, but all attempts made thus far to identify the active agents with this group have failed. On the other hand the anemia-producing substance of deaminized casein may be detoxicated by some non-essential amino acid, and thus the essential amino acids may not be concerned in the process. It may be also that there are unsuspected gaps in our knowledge of the constituent amino acids of casein and lactalbumin. Another possibility is that when deaminized casein is in the ration the animals require more of certain amino acids than they do when this protein is absent, and that the amino acids were offered in too small an amount to be effective. In future attempts to solve the problem chief reliance will be placed on a more complete fractionation of the active copper salts, combined with the use of increased amounts of amino acids, individually and in combination.

Since it now seems certain that this type of anemia can be prevented by certain of the amino acids, a solution of the immediate problem can be anticipated with some confidence. One can also anticipate that the solution will raise other questions which at this time seem equally puzzling. Casein is highly effective as the antianemic agent. The laboratory preparation of lactalbumin, wheat gluten, or a mixture of gelatin and gliadin is not effective. Our present knowledge of the constituent amino acids of these proteins does not suggest a satisfactory explanation for this difference.

SUMMARY

1. Of the proteins examined casein is the most potent source of the antianemic agent. A considerable portion of the activity remains after hydrolysis. Lactalbumin is relatively ineffective, and both corn and wheat gluten are still more deficient.
2. The antianemic agent is not extracted from hydrolyzed casein by butyl alcohol.
3. The antianemic agent forms a copper salt soluble in water but insoluble in methyl alcohol.
4. The anemic animals were not cured by supplying them with amino acids supposed to be in this copper salt fraction. Supplements of the individual essential amino acids which were not expected to be in this fraction were equally ineffective.
5. The spleens of anemic rats were much enlarged.

BIBLIOGRAPHY

1. Hogan, A. G., and Ritchie, W. S., *J. Biol. Chem.*, **107**, 179 (1934).
2. Hogan, A. G., Guerrant, R. E., and Ritchie, W. S., *J. Biol. Chem.*, **115**, 659 (1936).
3. Elvehjem, C. A., and Kemmerer, A. R., *J. Biol. Chem.*, **93**, 189 (1931).
4. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
5. Windus, W., Catherwood, F. L., and Rose, W. C., *J. Biol. Chem.*, **94**, 173 (1931-32).
6. Dakin, H. D., *Biochem. J.*, **12**, 290 (1918).
7. Caldwell, C. T., and Rose, W. C., *J. Biol. Chem.*, **107**, 57 (1934).
8. Jones, D. B., and Moeller, O., *J. Biol. Chem.*, **79**, 429 (1928).

9. Smith, M. I., and Stohlman, E. F., *Pub. Health Rep., U. S. P. H. S.*, **51**, 772 (1936).
10. Hamre, C. J., and Miller, C. D., *Am. J. Physiol.*, **111**, 578 (1935).
11. Donaldson, H. H., The rat, Memoirs of The Wistar Institute of Anatomy and Biology, Philadelphia (1915).
12. Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, **16**, 103 (1938).
13. Greaves, E. O., Morgan, A. F., and Loveen, M. K., *J. Nutrition*, **16**, 115 (1938).
14. Schmidt, C. L. A., and Allen, F. W., Fundamentals of biochemistry, New York (1938).

THE COMPOSITION OF THE TISSUE LIPIDS OF THE VIRGINIA WHITE-TAILED DEER (ODOCOILEUS VIRGINIANUS BOREALIS)

BY C. R. TREADWELL AND H. C. ECKSTEIN

(From the Laboratory of Biological Chemistry, Medical School, University of Michigan, Ann Arbor)

(Received for publication, February 9, 1939)

This report on the composition of the lipids extracted from six different tissues of the Virginia white-tailed deer (*Odocoileus virginianus borealis*) was made possible through the cooperation of the Department of Forest Zoology, School of Forestry, University of Michigan.[1] For some time this Department has been observing a herd of these animals living on the Edwin S. George Reserve, Museum of Zoology, located in Livingston County 24 miles northwest of Ann Arbor. The present herd is from a stock of four does and two bucks transported from Grand Island during the years 1926–27. These animals are all in good nutritional condition and their diet is one which most nearly simulates their natural one. A study of the lipids seemed desirable, since no record has been made of the chemical composition of the fatty substances of this species.

In our study the tissues from five animals were available. The deer were all shot on the same day during the winter of 1938. Two of the animals (Nos. 2 M. and 3 M.) were found to be infected with a lung parasite, but their remaining tissues were to all appearances normal. The tissues were removed as soon as possible after death, immediately placed in jars, covered with 95 per cent alcohol, and transported to Ann Arbor where they were stored in a refrigerator until analysis.

The lipids were extracted and prepared for analysis in the following manner. The alcohol was poured off from the sample and eventually combined with the subsequent extracts. The tissue was then cut into small pieces, ground with sand in a mortar under 95 per cent alcohol, and transferred to a flask with a liberal amount of 95 per cent alcohol. The mixture was then refluxed for 15 minutes, allowed to settle, and the supernatant liquid decanted. Refluxing was repeated three times with 95 per cent alcohol and once with absolute alcohol. The residue which now consisted mainly of connective tissue was again ground with sand and then extracted four times by refluxing with anhydrous ethyl ether for 5 minute periods. These alcohol and ether extracts were combined with the alcoholic solution originally poured off and concentrated *in vacuo* at 50-60° until practically all of the solvents had been distilled off. The oily residue was taken up in purified petroleum ether (b.p. 30-60°, iodine number 0). This petroleum ether solution was washed three times with water, dried over anhydrous sodium sulfate, and, after filtration, diluted to a definite volume with petroleum ether. Aliquots were used for the determination of total lipids, iodine number (Wijs), saponification number, thiocyanogen number, solid and liquid fatty acids,[2] and total sterols. Total lipids were determined in an aliquot transferred to a weighed flask. The solvent was removed by distillation *in vacuo* and the residue dried to constant weight in a vacuum desiccator over phosphorus pentoxide. The solution that remained after the saponification number had been estimated was used for total sterol determination. This residual solution was made distinctly alkaline with 5 cc. of 50 per cent sodium hydroxide and extracted four times in a separatory funnel with 75 cc. portions of ethyl ether. The combined ether extracts were washed with water until the aqueous extracts were neutral to phenolphthalein. These washings were extracted twice with ethyl ether and the extracts washed as above. The ether in the united extracts was distilled off *in vacuo* and the residue taken up in an equal mixture of absolute alcohol and anhydrous acetone. The total sterols were then determined photometrically by the method of Schoenheimer and Sperry (2). The oleic and

---

[1] We wish particularly to express our thanks to Professor E. C. O'Roke and Mr. F. C. Goble for their assistance in this matter.

[2] The procedures used for thiocyanogen number and solid and liquid fatty acid determinations are those recommended in a report of the Committee on Analysis of Commercial Fats and Oils, American Chemical Society (1).

linoleic glycerides were calculated from the iodine and thiocyanogen numbers.

TABLE I

Composition of Tissue Lipids of Virginia White-Tailed Deer

Source of lipids	Animal No. and sex	Saponification No.	I No.	Thiocyanogen No.	Glycerides of total lipids*			Fatty acids of total fatty acids†		I No. of solid acids	Total sterols of total lipids
					Linoleic	Oleic	Liquid	Solid	Liquid		
					per cent	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*
Pericardial	1 M.	194.8	29.0	26.7	2.7	28.3	31.0	64.1	35.9	4.5	0.18
	2 "	193.1	28.6	24.4	4.9	23.5	28.4	64.5	35.5	6.7	0.19
	3 "	191.0	29.8	27.3	2.9	28.8	31.7	65.4	34.6	5.5	0.18
	4 F.	196.7	29.0	26.2	3.2	27.2	30.4	64.1	35.9	3.0	0.12
Average......		193.9	29.1	26.2	3.4	26.9	30.4	64.5	35.5	4.0	0.18
Perirenal	1 M.	193.4	31.1	27.2	4.5	27.0	31.5	64.4	35.6	2.3	0.19
	2 "	192.7	29.7	25.1	5.3	23.8	29.1				0.21
	3 "	191.1	28.9	27.2	2.0	29.6	31.6	66.1	33.9	4.7	0.25
	4 F.	193.8	31.5	28.6	3.4	29.9	33.3	61.0	39.0	2.6	0.16
	5 M.	195.8	30.1	26.8	3.8	27.3	31.1	55.4	44.6	5.9	0.19
Average......		193.4	30.3	27.0	3.8	27.5	31.3	61.7	38.3	3.9	0.20
Omental	3 M.	192.1	34.4	28.6	6.7	26.5	33.2				0.17
	4 F.	194.6	32.7	30.1	3.0	31.9	34.9	58.0	42.0	3.4	0.14
Average......		193.3	33.6	29.4	4.9	29.2	34.1	58.0	42.0	3.4	0.16
Mesenteric	2 M.	192.6	33.1	30.1	3.6	31.5	35.1	62.7	37.3	6.7	0.19
Pericecal	4 F.	193.3	27.4	25.4	2.3	27.2	29.5	63.4	36.6	3.8	0.17
Mammary	4 "	195.0	40.6	38.9	2.0	43.2	45.1				0.14
General average‡...		193.5	30.4	27.2	3.7	27.9	31.6	62.6	37.4	4.5	0.18

* Calculated from iodine and thiocyanogen numbers.

† Calculated from lead salts.

‡ The values for mammary tissue were omitted in obtaining the general average.

The data obtained are shown in Table I. It is evident from that summary that, with the exception of those in the mammary gland, the tissue lipids are quite similar in composition. There are some exceptions particularly in the case of the linoleic glycerides but even these values are all of the same order of magnitude. In the general average, the data on the mammary tissue are excluded, because the lipids therein are not considered to be depot fats, as is the case for the other tissue lipids. The following summation will be limited to the general averages. Those obtained for saponification, iodine, and thiocyanogen numbers were 193.5 (191 to 197),[3] 30.4 (27.4 to 34.4), and 27.2 (24.4 to 30.1) respectively. The total lipids contained 3.7 (2.0 to 6.7) per cent linoleic, 27.9 (23.5 to 31.9) per cent oleic, and 31.6 (28.4 to 35.1) per cent liquid glycerides. 0.18 (0.12 to 0.25) per cent of the total lipids was in the form of total sterols and 62.6 (55.4 to 66.1) per cent and 37.4 (33.9 to 44.6) per cent of the total fatty acids were respectively solid and liquid. It is evident from the iodine numbers of the solid fatty acids that the determination of these by the lead salt method was satisfactory. The saponification number and total sterol content of the mammary lipids were similar to those obtained for the other tissue lipids. The iodine and thiocyanogen numbers as well as the oleic glyceride contents were, however, all higher than the values recorded for the others. Too much emphasis should not be placed on these differences, since only one sample of mammary tissue was available.

This general tendency towards a uniform composition of the different kinds of reserve lipids of our deer is in line with the observations of Reed, Yamaguchi, Anderson, and Mendel (3) who reported that the distribution of the depot fat of the white rat was not influenced by the diet and that the degree of saturation of the lipids in the intermuscular, subcutaneous, perirenal, mesenteric, and omental tissues of the rat was essentially the same. Henriques and Hansen (4) found little difference between the reserve fats of the dog or the duck, but recorded a dissimilarity between the depot fats of the hog. The iodine numbers of our deer lipids are like those reported by Amthor and Zink (5) for the roebuck (25.7) and for the elk (35). Gadpole and Sadgopal (6) using the same method of calculation employed by us give values of 5.18 per cent linoleic and 35.58 per cent oleic glycerides for beef tallow and

---

[3] The values in parentheses are the variations obtained.

6.85 per cent and 32.02 per cent respectively for mutton tallow. These values are like those recorded by us in Table I.

This project was made possible by a grant from the Faculty Research Fund, administered through the Executive Board of the Horace H. Rackham Graduate School of the University of Michigan.

SUMMARY

The perirenal, pericardial, pericecal, omental, and mesenteric lipids of the Virginia white-tailed deer (*Odocoileus virginianus borealis*) were found to be quite uniform in composition. The average values for saponification, iodine, and thiocyanogen numbers were 193.5 (191 to 197), 30.4 (27.4 to 34.4), and 27.2 (24.4 to 30.1) respectively. The total lipids contained 27.9 (23.5 to 31.9) per cent oleic glycerides, 3.7 (2.0 to 6.7) per cent linoleic glycerides, 31.6 (28.4 to 35.1) per cent liquid glycerides, and 0.18 (0.12 to 0.25) per cent total sterols. 62.6 (55.4 to 66.1) per cent and 37.4 (33.9 to 44.6) per cent were respectively solid and liquid fatty acids.

The single sample of the total lipids of the mammary gland had a saponification number of 195, an iodine number of 40.6, and a thiocyanogen number of 38.9. These lipids contained 43.2 per cent oleic, 2.0 per cent linoleic, and 45.1 per cent liquid glycerides. The total sterol content of the total lipids was 0.14 per cent.

BIBLIOGRAPHY

1. Irwin, W. H., Bailey, R. W., Law, T. C., Long, C. P., Morrison, H. J., Sheely, M. L., Tolman, L. M., Trevithick, H. P., and Vollersten, J. J., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 33 (1936).
2. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
3. Reed, L. L., Yamaguchi, F., Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **87**, 147 (1930).
4. Henriques, V., and Hansen, C., *Skand. Arch. Physiol.*, **11**, 151 (1901-02).
5. Amthor, C., and Zink, J., *Z. anal. Chem.*, **36**, 1 (1897).
6. Gadpole, N. N., and Sadgopal, *Allg. Oel- u. Fett-Ztg.*, **31**, 435 (1934); *Chem. Abst.*, **29**, 4959 (1935).

EFFECT OF CARBOHYDRATE AND FAT IN THE DIET ON URIC ACID EXCRETION

By DAVID ADLERSBERG and MAX ELLENBERG

(From the Medical Service of Dr. George Baehr and the Laboratories of the Mount Sinai Hospital, New York)

(Received for publication, February 16, 1939)

Apart from an early observation by Umeda (1) of a single case in which a diet high in fat and low in carbohydrate led to a diminution of uric acid output, little attention was devoted to this phase of the uric acid problem prior to the introduction of the ketogenic diet in the treatment of epilepsy. This stimulated the study of the uric acid elimination during fasting in epileptic children, normal persons being used as controls. All observers (2–6) reported that fasting resulted in decreased uric acid elimination in the urine. Leopold, Bernhard, and Jacobi (4) further showed that a high fat diet results in a marked decrease in the uric acid output. Their diet, used in children, was unusually low in carbohydrate, containing, daily, only 15 gm. of carbohydrate, 12 gm. of protein, and 110 gm. of fat. They also noted that a high carbohydrate, almost purine-free diet does not result in greater uric acid elimination than that observed on a diet with an equally low purine content, and moderate amounts of carbohydrate. Lennox (5), in a very comprehensive paper, showed that the feeding of a high fat diet does not relieve the uric acid retention resulting from prolonged fasting, but that the feeding of carbohydrate results in a prompt rise in uric acid excretion.

A state of acidosis and ketosis may be induced by a high fat, extremely low carbohydrate diet, as well as by prolonged fasting. The first may be considered an "exogenous" high fat diet, whereas the effect of fasting is essentially equivalent to an "endogenous" high fat diet. The diminished excretion of uric acid under both these conditions may therefore have been due to acidosis or ketosis. In fact, Lennox (5) demonstrated a fairly reciprocal relationship between the blood uric acid and the plasma bicarbonate in the course of his fasting experiments. To rule out acidosis as distinguished from ketosis, he added large amounts of sodium bicarbonate but there was no increase in the output of uric acid. He also produced an acidosis, without ketosis, by administering calcium chloride but caused no change in the uric acid excretion. These observations excluded acidosis *per se* as the factor responsible for the diminution in uric acid excretion during fasting or with high fat diets. It was still to be determined whether the high fat diet itself or the accompanying ketosis was responsible for the diminution of uric acid excretion.

A review of the literature did not reveal any studies on uric acid excretion that differentiated the effect of the high fat diet from that of the accompanying ketosis in normal individuals. We attempted to determine this by giving high fat diets which, however, included sufficient carbohydrates to prevent ketosis.

Procedure and Method

Seven patients were used as subjects. They were all afebrile and well along in convalescence from various diseases, none of which was related to uric acid metabolism. These subjects were first placed on a diet of 150 gm. of carbohydrate, 80 gm. of protein, and 100 gm. of fat (Diet I), to determine the average daily uric acid excretion for each individual. This was followed by a diet of 150 gm. of carbohydrate, 80 gm. of protein, and 250 gm. of fat (Diet II). Diet II represented an increase of 150 gm. of fat over that contained in Diet I, while the carbohydrate and protein content remained unchanged. The subjects were then returned to the basal diet of 150 gm. of carbohydrate, 80 gm. of protein, and 100 gm. of fat (Diet III). Three of the subjects were subsequently given a fourth diet (Diet IV) which contained 350 gm. of carbohydrate in addition to the basal diet and was practically equivalent in calories to Diet II. The actual caloric values of the four types of diets were Diet I 1850, Diet II 3200, Diet III 1850, and Diet IV 3250 calories.

Each day the total 24 hour urine was collected, with toluene as a preservative, measured, and analyzed for its uric acid content. The uric acid content was determined by Brown's modification of the Benedict method (7). With each change of diet, the uric acid of the blood serum was determined by the method of Benedict. During administration of the high fat diet (Diet II) frequent tests for acetonuria were performed. Occasional determinations of the carbon dioxide-combining power of the blood were also made.

Results

Table I presents the uric acid excretion of one of the subjects on the varied diets administered. On the basal diet (Diet I), the daily uric acid excretion ranged between 505 and 567 mg., the average being 532 mg. On Diet II, which is rich in fat, the daily excretion varied from 300 to 480 mg., the average being 408 mg. This represented a 23 per cent reduction in uric acid excretion from that on the basal diet. A return to the basal diet, Diet III, resulted in a daily excretion ranging from 510 to 600 mg. of uric

TABLE I

Effect of High Fat and of High Carbohydrate Diets on Excretion of Uric Acid. Case 1, E. N.

	Day	Urine		Blood uric acid
		Amount	Uric acid	
		cc.	*mg.*	*mg. per cent*
Diet I, 150 gm. carbohydrate, 80 gm. protein, 100 gm. fat	1	1490	552	
	2	1120	515	
	3	1200	567	1.9
	4	1400	505	
Diet II, 150 gm. carbohydrate, 80 gm. protein, 250 gm. fat	5	700	300	
	6	1350	450	
	7	1450	430	
	8	1400	480	2.2
	9	1460	382	
Diet III, same as Diet I	10	1510	600	
	11	1820	510	
	12	1540	568	1.8
Diet IV, 500 gm. carbohydrate, 80 gm. protein, 100 gm. fat	13	1600	550	
	14	1400	500	
	15	1500	600	
	16	2400	658	

acid, the average being 559 mg., which is only slightly elevated as compared with the first period. On Diet IV, which is high in carbohydrates, and of approximately the same caloric content as the high fat diet (No. II), the uric acid excretion ranged from 500 to 658 mg., the average being 584 mg. This excretion is slightly greater than that observed with Diet III and is moderately elevated as compared with that of the original period on the basal diet (Diet I).

Table II presents the average amounts of uric acid excretion of all subjects during each diet period. The average daily uric acid excretion on Diet I varied from 495 to 717 mg., thus indicating a

TABLE II

Average Daily Excretion of Uric Acid in Each Diet Period

Case	Diet I, basal		Diet II, high fat		Diet III, basal		Diet IV, high carbohydrate		Decrease between Diet I and Diet II
	Days on diet	Uric acid	Days on diet	Uric acid	Days on diet	Uric acid	Days on diet	Uric acid	
		mg.		*mg.*		*mg.*		*mg.*	*per cent*
E. N.	4	532	5	408	3	559	4	584	23
M. U.	3	717	5	527	5	673	4	687	28
R. E.	3	523	8	414	5	488	6	544	21
C. S.	3	617	7	510	1	506			17
E. M.	5	511	9	443	5	354			11
L. M.	7	608	6	454	3	555			25
N. G.	9	495	7	377					24

wide range for different individuals, but the daily excretion in each individual ranged within much narrower limits. During the period on Diet II (high fat) the average daily uric acid excretion decreased in each case from 11 to 28 per cent. The average decrease for all seven subjects was 21.3 per cent as compared with the basal diet (Diet I). A return to the low fat diet (Diet III) was followed in each of the cases, except one, by an increase in the daily uric acid excretion, the average value being moderately decreased as compared with the period of Diet I.

Only three of our subjects were put on Diet IV. On this diet the average daily uric acid excretion was 605 mg., while the average daily uric acid excretion of these three subjects on Diet I was

590 mg., on Diet II 449 mg., and on Diet III 573 mg. While there was a very definite difference in uric acid excretion during the administration of the high carbohydrate and the high fat diet, a diminution of 25.8 per cent on the latter, there were only slight differences during the two periods on the basal diet and the carbohydrate diet.

The duration of each period varied from 1 to 9 days. In all cases frequent examinations of the urine for acetone during the period on Diet II failed to reveal the presence of acetone bodies. The occasional determinations of the blood carbon dioxide-combining power revealed normal values. At times there was a slight elevation of the blood uric acid during the administration of high fat diet, as in Table I. However, this was not consistent and usually varied within the range of error of the method used.

DISCUSSION

The classical treatment of gout by means of a diet low in proteins and purines is still the customary basis of therapy. However, recent investigations have revealed the importance of the relative amounts of carbohydrate and fat, as well as of protein, in the diet. Earlier investigations, as outlined above, indicated that a diet high in fat and low in carbohydrate caused a diminution in the excretion of uric acid in the urine, and that this diminution was not due to the acidosis developed. Our experiments have further shown that neither the ketosis nor the high caloric value of the diet is the cause, and that the result appears to depend directly on the high fat content.

The mechanism underlying these changes in the uric acid excretion is not determined. The observed diminution might be due to (1) diminished function of the kidney, (2) decreased absorption of purines from the diet due to a change in the amount of destruction in the intestines, (3) diminished "endogenous" production of purines, (4) disturbance of the function of the liver in formation of uric acid, or (5) increased retention in the tissues. The first was suggested by the earlier investigators, who considered the change as a "retention," but our data do not indicate a significant rise in blood uric acid. The second appears to us unlikely, in view of the low purine content of the diet. On the third, we have no information. The fourth (diminished formation by the liver) seems at least plausible, since the influence of high fat diet and high carbohydrate diet on some functions of the liver is well known and has received extensive therapeutic application (as, for example, in diseases of the liver parenchyma). The fifth possibility (increased retention by the tissues) cannot be excluded, especially in view of the clinical effect of a high fat diet in causing an exacerbation of symptoms in gouty individuals. This effect might also argue against the occurrence of decreased uric acid formation as the cause of diminished excretion, since it seems unlikely that a decrease in production would bring on the symptoms.

The effect of a high fat diet on patients with gout was studied by Lockie and Hubbard (8). Each of four gouty patients whose symptoms had subsided was placed on a very high fat, low carbohydrate diet. This was accompanied by a prompt return of symptoms, as well as by a reduction in the uric acid output and a rise in the blood uric acid. These patients were then put on a high carbohydrate, low-fat diet, with a prompt relief in symptoms. Their studies are open to criticism, since their high fat diets were very low in carbohydrate, containing only 20 to 60 gm. However, the high carbohydrate diet which produced such beneficial effects varied in its content of fat and contained, in one instance, 130 gm. of fat per day. As soon as adequate clinical material has been obtained, we shall submit a report upon observations with similar diets on gouty patients.

This clinical observation plus our results may have a direct bearing on the therapy of the gouty diathesis which many clinicians believe to be more wide-spread than is commonly supposed. It would appear, therefore, that the diet in gouty patients should be low in purines to control the extrinsic factor, and low in fat content to control the intrinsic factors responsible for uric acid elimination. The resulting diet would therefore necessarily be high in carbohydrate content, with only moderate amounts of protein and fat. Mention may be made of the not infrequent association of gout and diabetes. The dietetic problem in these cases should now be greatly simplified, since our conclusions point to the value of a high carbohydrate diet in gout and the present tendency in diabetes is to employ a relatively high carbohydrate diet particularly with the use of insulin.

SUMMARY

1. A high fat diet diminishes the uric acid excretion in normal individuals.

2. Neither acidosis nor ketosis is of importance in this connection, since these factors were controlled by sufficient amounts of carbohydrate in the diet.

3. A high carbohydrate diet, calorically equivalent to the high fat diet, does not depress the uric acid elimination.

4. The factor responsible for the decrease in uric acid excretion in the urine of subjects on a high fat diet is not the increased caloric intake, but is the high fat content itself.

5. These observations support the use of a high carbohydrate, low fat, as well as a low purine diet in the treatment of the uric acid diathesis.

We are greatly indebted to Dr. Harry Sobotka under whose direction and supervision the urine and blood analyses were carried out.

BIBLIOGRAPHY

1. Umeda, N., *Biochem. J.*, **9**, 421 (1915).
2. Hoeffel, G., and Moriarty, M., *Am. J. Dis. Child.*, **28**, 17 (1924).
3. Lennox, W. G., *J. Am. Med. Assn.*, **82**, 602 (1924).
4. Leopold, J. S., Bernhard, A., and Jacobi, H. G., *Am. J. Dis. Child.*, **27**, 243 (1924).
5. Lennox, W. G., *J. Biol. Chem.*, **66**, 521 (1925).
6. Harding, V. J., Allin, K. D., Eagles, B. A., and Van Wyck, H. B., *J. Biol. Chem.*, **63**, 37 (1925).
7. Brown, H., *J. Biol. Chem.*, **68**, 123 (1926).
8. Lockie, L. M., and Hubbard, R. S., *J. Am. Med. Assn.*, **104**, 2072 (1935).

THE LIVER LIPIDS OF DOGS SUBJECTED TO LIGATION OF THE EXTERNAL PANCREATIC DUCTS*

By M. L. MONTGOMERY, C. ENTENMAN, and I. L. CHAIKOFF

(From the Division of Surgery, University of California Medical School, San Francisco, and the Division of Physiology, University of California Medical School, Berkeley)

(Received for publication, February 20, 1939)

Neutral fat in large amounts appears in the livers of completely depancreatized dogs kept alive with insulin and a diet which, though lacking in pancreas, contains an excess of all constituents essential for maintenance of the normal dog (1, 2). A time factor has been shown to exist in the development of the lipid deposits. Although as much as 32 per cent fatty acids may appear in the liver as early as 3.5 weeks after pancreatectomy, approximately 20 weeks were necessary to establish livers containing an excess of 14 per cent fatty acids in a large series of dogs. These fatty deposits apparently remain for long periods thereafter and in a single dog a fatty liver was noted as late as 3 years after pancreatectomy. A spontaneous decline in the fat content of the liver may occur, however, if the animal survives long enough. In three dogs that had survived for 4.2 to 5.5 years, approximately normal percentages of fatty acids were found, although the total amount of fatty acids present was still in excess of the normal, owing to the fact that the size of the liver failed to regress as the fat left it.

The mechanism for the production of these fatty livers is still obscure. Certain factors that influence this condition have been determined. Both pancreas and choline have been shown to possess curative and preventive actions (2–4). The curative effect of both was found to be slow; a feeding period in excess of 16 weeks was required to restore a fatty liver to normal. The

---

* Aided by grants from the Christine Breon Fund for Medical Research and the Melville Luther Montgomery Donation. Assistance was also furnished by the Works Progress Administration (Official Project No. 665-08-3-30, Unit A6).

active factor (or factors) is heat-stable, for after being autoclaved at 20 pounds pressure for 30 minutes pancreas was still found to possess preventive and curative actions (2). This suggests that the factor is not enzymatic in nature. Recently Dragstedt *et al.* (5) claimed there exists an internal secretion of the pancreas having to do with the control of fat metabolism in the liver, the absence of which explains the occurrence of fatty livers in depancreatized dogs. But their failure to keep their depancreatized dogs alive for any length of time has led to criticism of their work (6). Their use of histological examination of microscopic sections instead of chemical analyses for fat determinations is open to question, the more so since it was found in this laboratory that fat is not necessarily distributed equally throughout livers containing it in moderate amounts (7). Finally, they did not rule out adequately the curative effect of the choline in their extract before claiming that it contained a new hormone.

Although the above findings refer to the fatty liver only of the depancreatized dog, a number of workers have employed normal rats fed high fat diets in an attempt to solve this problem. But it has been pointed out elsewhere that, although choline affects similarly the fatty livers of rats fed a *high fat, low protein diet* and those of depancreatized dogs fed a *low fat, high protein diet*, the evidence available at present does not permit the conclusion that the mechanism whereby these two types of fatty liver occur is the same (3). It may not be admissible to employ an animal already supplied with pancreatic juice and an intact pancreas in experiments designed to test the presence or absence of a new pancreatic factor (hormone). Moreover, so far as these investigations (8–10) have gone, they have yet to show uniformly that the effects of an extract of the pancreas upon the fatty liver of the normal rat cannot be explained by its choline and protein content.

In order to investigate the mechanism for the production of fatty livers in depancreatized dogs, it seemed desirable at the outset to determine the relation of the deposition of fat to the external secretion of the pancreas, since pancreatectomy, in addition to removing all islet tissue, completely eliminates the pancreatic juice from the intestinal tract. Pancreatic juice can be excluded either by fistula or by duct ligation. The interpretation of experiments in which the pancreatic juice was removed by a fistula is beset with difficulties, since no reports have yet appeared in which the animals were maintained in a satisfactory state for long periods. In 1935 Aubertin *et al.* (11) reported fatty livers in three dogs subjected to ligation of the pancreatic ducts, and this finding has been more recently confirmed by Ralli *et al.* (12) and by Person and Glenn (13). On the other hand Dragstedt and his coworkers (14) concluded that fatty livers do not occur in dogs subjected to this surgical procedure.

In the present investigation the effects of ligation of the pancreatic ducts upon the lipid content of the liver of the dog are studied. It is shown that complete ligation of the ducts produces fatty livers, provided that sufficient time is allowed to elapse after the operation. It is also shown that the deposition of abnormal amounts of lipids observed under these conditions can appear in the absence of weight loss and can be completely inhibited by the addition of raw pancreas to the diet.

EXPERIMENTAL

Method of Ligation of Pancreatic Ducts—Dogs were used throughout this investigation. With aseptic precautions the two main pancreatic ducts were exposed under general anesthesia, doubly ligated, and divided between the ligatures. A second ligature was placed around the stump of each duct on the pancreatic side of the division, and by means of this ligature the duct stump was drawn through a tunnel prepared in the gland and ligated to the mesenteric attachment of the pancreas. This procedure was designed to prevent the reestablishment of the external secretory system of the pancreas with the duodenum. It was successful in all but one of the twenty dogs.

A meticulous dissection was made of all connections of the pancreas with the duodenum from the region of the pylorus to a point at least 2 cm. below the location of the lower pancreatic duct, at which level the gland is separated from the bowel by a distance of 1.5 to 2.0 cm. These attachments were scrutinized carefully, and all but the major blood vessels were divided. All possible ductal communications were thereby interrupted. As a further precaution against ductal recanalization the pancreas and duodenum were kept apart by rather large tongues of omentum, which were pulled in through the arches formed by the attached vessels and made to fill the space left by the dissection of the pancreas from the duodenum. These omental tongues were then sutured in place.

Care and Maintenance of Animals—All dogs selected possessed excellent appetites. Before the operation they were brought into good nutritional state by means of a diet high in lean meat, vitamins, and bone ash. Food was withheld for 1 day before and 1 day after the operation. The animals recorded below experienced no loss in appetite as a result of ligation of the ducts; they continued to eat all food placed before them. They were fed twice daily, at 8.00 a.m. and 4.00 p.m. In view of the impaired absorption resulting from the absence of pancreatic juice, considerable attention was paid to the vitamin content of the diet. All dietary mixtures were supplemented with vitamin sources: A and D as cod liver oil,[1] the vitamin B complex[2] in the form of a concentrate obtained from rice bran. This concentrate contained about 50 I. U. of vitamin B (B_1) per cc. and 10 modified[3] Bourquin-Sherman units of vitamin G (flavin) per cc.; the same concentrate has also been shown to be a good source of both rat and chick antidermatitis factors (15) and the P-P factor. Each gm. of the cod liver oil contained not less than 1800 vitamin A units, U.S.P., and not less than 175 vitamin D units, U.S.P. Salts were added to the diet either as bone ash or as Cowgill's salt mixture (16).

Livers were taken for analyses between 8.00 and 9.00 a.m.; the dogs had received their last meal (or last meal and injection of insulin) at 4.00 p.m. of the previous day. This state of the animal in which it has been deprived of both food and insulin for 16 hours is hereafter referred to as the postabsorptive state.

The liver was removed after the animal had been anesthetized with sodium amytal. A mixed sample of the *whole* liver was used for lipid estimation; the methods of sampling and of lipid estimations have been previously described (1). Blood sugar was determined with the copper-iodometric reagent of Shaffer and Somogyi

---

[1] The standardized cod liver oil was kindly furnished by Mead Johnson and Company.

[2] The vitamin B concentrate was kindly furnished by Vitab Products, Inc., Emeryville, California.

[3] The basal diet for rat assay was modified to supply the rat and chick antidermatitis factors.

(17), while the filtrate was obtained by the precipitation of blood with zinc hydroxide (18).

Examination for Completeness of Duct Ligation—It was stated by Macleod, in reviewing earlier work (19), that "the chief objection to duct ligation investigations depends on the uncertainty that the secretory pathway has really been permanently blocked." Hence in the present study great care was exercised to insure complete severance of all communications between pancreatic ducts and duodenum. Of the twenty dogs used only one (Dog E-27[4]) showed recanalization; this was recognized by the persistence of grossly evident acinous tissue and by the fact that dissection showed a very minute but definite communication at the site of the lower pancreatic duct. In all the nineteen dogs recorded in Tables I to III, the completeness of duct ligation was confirmed by careful inspection and dissection at necropsy; the characteristic appearance of acinous tissue was absent and the gland was greatly atrophied. In all nineteen animals the remnant of pancreatic tissue found at the end of the period of observation was characterized by absence of the pink-yellow color of the normal gland. Instead, the residual structure showed fine to moderately coarse lobulations arranged radially around the pancreatic ducts in the middle half of the gland and spread out at either end as a fan-like process within the mesentery. These elements were more deeply colored than the normal gland and in many instances had a semi-translucent appearance. It was also observed that the glandular tissue that remained was usually much more abundant in the proximal third of the gland. The least amount of glandular elements was found in the medial third. The amount of gland found in the distal third was quite variable. At no place in the fanned-out areas was glandular tissue found to be thicker than 1.5 mm.

Results

Liver Lipids of Duct-Ligated Dogs Maintained on Lean Meat Diet—The lipid constituents found in the livers of nine dogs maintained on a high meat diet (with or without the addition of sucrose) for varying periods after duct ligation are shown in Table I. Three livers obtained from animals at intervals of 21 to 24 weeks after the operation contained 11.4, 13.5, and 29 per cent fatty acids. Three other dogs were examined 16 to 18.5 weeks after ligation; their livers contained 18 to 21 per cent fatty acids. The other three were maintained for 12 to 14.5 weeks after the operation; of these three the livers of Dogs E-13 and E-20 contained respectively 11.5 and 35.5 per cent fatty acids, whereas the liver of Dog E-23 showed a normal lipid content; namely, 2.7 per cent fatty acids. Thus, eight of the nine dogs in which the pancreatic

---

[4] Normal amounts of fatty acids were found in the liver of this animal.

TABLE I

Liver Lipids of Duct-Ligated Dogs Maintained on Lean Meat (with or without Sucrose) Diet*

Dog No. and sex	Weight		Period maintained after duct ligation	Liver weight	Lipids			Diet†		Condition at end of period of observation‡
	Preoperative	Final			Total cholesterol	Total fatty acids	Phospholipids	Lean meat	Sucrose	
	kg.	*kg.*	*wks.*	*gm.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*	
E-7 ♀	11.6	8.0	24	360	0.41	11.4	1.75	165	35	Thin
E-9 ♀	9.0	5.8	23	310	0.62	13.5	2.04	135	27	Weak and emaciated
E-12 ♀	9.6	8.9	21	370	0.66	29.0	1.41	250	50	Good nutritional state
E-13 ♂	11.0	8.6	14.5	290	0.41	11.5	2.04	250	50	Weak
E-20 ♂	12.0	6.6	12	510	0.32	35.5	1.93	305		Very weak and emaciated
E-23 ♀	15.0	6.7	13.5	140	0.51	2.67	2.48	350		" "
E-29 ♀	10.5	8.4	18.5	220	0.31	17.7	2.06	282		Good condition
E-31 ♀	15.8	8.1	18.5	300	0.42	20.6	1.79	362		Very weak at end
E-34 ♂	11.4	5.0	16	255	0.36	19.6	1.84	295		Extremely weak and emaciated

* Completeness of ligation of ducts confirmed at necropsy.

† The constituents in amounts recorded were fed twice daily at 8.00 a.m. and at 4.00 p.m. Vitamin supplements were added either daily or twice weekly. Each animal received approximately 25 cc. of cod liver oil and 30 cc. of the vitamin B concentrate per week. 2 gm. of Cowgill's salt mixture (15) were added daily.

‡ All the dogs showed good appetites throughout the period of observation.

juice had been excluded from the intestinal tract showed a fat content in their livers greater than normal.

All dogs recorded in Table I showed a loss in weight after duct ligation despite the fact that each maintained a vigorous appetite throughout, ingesting rapidly the large amounts of food placed before it. Dogs in which the weight loss was particularly marked suffered from muscular weakness toward the end of the period of observation.

The sugar content of the blood was repeatedly determined in these dogs while in the postabsorptive state. With the exception of Dog E-20, the fasting blood sugar values fluctuated between 62 and 92 mg. per cent. Sugar levels of 75 to 82 mg. per cent were found in Dog E-20 during the first 9 weeks after duct ligation; a day prior to removal of the liver Dog E-20 showed a blood sugar of 127 mg. per cent.

Liver Lipids of Duct-Ligated Dogs Maintained on a Meat-Sucrose Diet and Insulin[5]—Marked atrophy of the pancreas was observed in all dogs when examined several months after duct ligation. While it is generally agreed that the acinous tissue rapidly degenerates after duct ligation, conflicting reports have appeared as to whether similar changes occur in the islands of Langerhans. Atrophic changes have been reported in the islet tissues as a result of duct ligation. It is important to note, therefore, that considerable degeneration of islet tissue could occur without affecting the fasting blood sugar, since it was shown many years ago that a large part of the pancreas has to be removed in order to induce diabetes in the dog (20). Hence the normal blood sugars recorded above do not necessarily rule out some possible islet changes in the atrophied pancreas. Since it is not inconceivable that different levels of the antidiabetic hormone may be required to control the carbohydrate and fat metabolism, it seemed desirable to control the possible lack of insulin in duct-ligated dogs by administering it. In Table II are shown the liver lipids of four dogs which had received twice daily 8 units of insulin in addition to a diet containing lean meat, bone ash, sucrose, and vitamin supplements. The insulin was injected at the same time that the dogs were fed the dietary mixtures. It has previously been established that this amount of insulin is sufficient to prevent the deposition of abnormal amounts of lipids in the depancreatized dog when pancreas is present in the diet (2).

---

[5] The insulin was generously donated by Eli Lilly and Company.

The results (Table II) show that the administration of 8 units of insulin twice daily failed to inhibit the abnormal deposition of lipids in the livers of these dogs. The livers of three animals contained 14.7, 20.4, and 34.2 per cent fatty acids. The liver of the fourth (Dog E-25) contained 6.3 per cent. In previous reports it was found that the livers of normal dogs contained 1.8 to 2.3 per cent fatty acids (1).

TABLE II

Liver Lipids of Duct-Ligated Dogs Maintained with Insulin and Lean Meat-Sucrose Diet*

Dog No. and sex	Weight		Period maintained after duct ligation	Liver weight	Lipids		
	Preoperative	Final			Total cholesterol	Total fatty acids	Phospholipids
	kg.	*kg.*	*wks.*	*gm.*	*per cent*	*per cent*	*per cent*
E-21 ♀†	5.0	6.7	20	255	0.28	14.7	1.60
E-22 ♀†	5.0	5.6	20	275	0.39	20.4	1.69
E-25 ♀†	9.0	7.6	20	185	0.32	6.32	1.98
E-30 ♂‡	13.5	12.3	16	470	1.03	34.2	1.85

* All dogs were in good nutritional state during the entire period of observation; their appetites were good throughout. Completeness of ligation of ducts confirmed at necropsy.

† Received twice daily 250 gm. of lean meat, 25 gm. of sucrose, and 7 gm. of bone ash. Vitamin supplements added twice weekly. Each dog received approximately 25 cc. of cod liver oil and 30 cc. of the vitamin B concentrate per week.

‡ This dog received 250 gm. of lean meat, 25 gm. of sucrose, and 7 gm. of bone ash twice daily for the first 6 weeks after duct ligation. The sucrose was then increased to 50 gm. twice daily. 7 weeks later the sugar was reduced to 25 gm. twice daily and this amount was fed the rest of the period of observation. During the last week convulsions appeared on three occasions. Low blood sugars were found at these times.

A striking feature of the four dogs recorded in Table II is the absence of the marked loss in weight that was observed in the dogs recorded in Table I. The weights of Dogs E-25 and E-30 fell from a preoperative level of 9.0 and 13.5 to 7.6 and 12.3 kilos 20 and 16 weeks respectively after duct ligation. Dogs E-21 and E-22 showed no weight loss at all during the period of observation after duct ligation; indeed the former gained 1.7 kilos after the operation. As noted above, the complete ligation of the ducts in these four dogs was confirmed at necropsy.

Postabsorptive blood sugars were repeatedly examined in these dogs during the period of observation following duct ligation. The blood sugar in Dogs E-21, E-22, and E-25 fluctuated between 66 and 97 mg. per cent. Dog E-30 was more sensitive to insulin, for blood sugars as low as 35 and 45 mg. per cent were observed at two intervals after duct ligation. It was this sensitivity to the hormone that led to the temporary increase in the carbohydrate of the diet recorded for this dog in the foot-note of Table II.

TABLE III

Liver Lipids of Duct-Ligated Dogs Maintained on Lean Meat-Raw Pancreas Diet*

Dog No. and sex	Weight		Period maintained after duct ligation	Liver weight	Lipids			Diet†	
	Preoperative	Final			Total cholesterol	Total fatty acids	Phospholipids	Lean meat‡	Raw pancreas
*	*kg.*	*kg.*	*wks.*	*gm.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*
E-15 ♀	17.7	18.9	20	345	0.24	1.87	1.82	265	125
E-17 ♀	9.0	8.9	20	220	0.25	2.28	2.18	135	125
E-19 ♂	7.8	8.9	20	220	0.26	3.10	2.71	117	125
E-24 ♀	10.5	10.0	20	270	0.27	2.84	2.78	157	125
E-33 ♂	14.4	15.3	20	285	0.24	3.49	2.33	215	125
E-28 ♂	8.9	8.3	22	270	0.23	2.90	2.55	133	125

* All dogs were in good nutritional state during period of observation; appetites good throughout. Completeness of ligation of ducts confirmed at necropsy.

† The constituents listed were fed twice daily. Vitamin supplements and 2 gm. of Cowgill's salt mixture were added daily. Each dog received approximately 25 cc. of cod liver oil and 30 cc. of the vitamin B concentrate per week.

‡ Amount of lean meat given was based on initial weight of dog: 30 gm. of lean meat per kilo per day.

Liver Lipids of Duct-Ligated Dogs Maintained on Diet of Lean Meat Plus Raw Pancreas—The dogs recorded in Table III received 125 gm. of raw pancreas twice daily in addition to the dietary mixture of meat, salt, and vitamins. The livers examined 20 weeks after all pancreatic secretions had been excluded from the intestinal tract contained normal amounts of total fatty acids;

namely, 1.9 to 3.5 per cent. Thus the ingestion of the raw glandular tissue completely inhibited the deposition of abnormal amounts of fat in the livers of these duct-ligated dogs. It is interesting to note that the ingestion of pancreas prevented the marked loss in weight observed in the dogs of Table I. In three of the six dogs of Table III distinct gains were observed 20 weeks after ligation of the ducts.

It has previously been shown that between 0.25 and 0.23 per cent total cholesterol is present in the livers of normal dogs (1). The livers of duct-ligated dogs in which the lipid infiltration had been prevented by the ingestion of raw pancreas contained approximately the same amount; namely, 0.23 to 0.27 per cent of the wet weight of the tissue (Table III). An increase in total cholesterol was observed in the fatty livers shown in Tables I and II. This increase occurred mainly in the esterified portion of the cholesterol, as was previously noted for the depancreatized dog (1,.2). Marked variations in phospholipid content of the liver were not observed in the dogs maintained under the various conditions shown in Tables I to III.

DISCUSSION

Fatty livers are produced in dogs by ligation of the pancreatic ducts, a procedure that excludes the external secretion from the intestinal tract. A variability in the amount of lipids deposited was noted, and in this respect the duct-ligated dog resembles the completely depancreatized dog maintained with insulin (2). Despite the extreme emaciation observed in some of the dogs recorded in Table I, there is no reason for believing at present that the fatty livers are the result of loss in weight, since fatty livers were also found in dogs in which either a slight loss or an actual gain in weight was observed as late as 20 weeks after ligation of all the pancreatic ducts (Table II). As previously noted for the completely depancreatized dog (2), the fattiness of liver produced by duct ligation is completely prevented by the addition of raw pancreas to the diet. The similarity of this action of pancreas in the duct-ligated dog and in the completely depancreatized dog maintained with insulin suggests that the mechanism whereby fatty livers are produced in the two types of animal preparations is the same.

While no complete explanation can be offered at present for the deposition of fat in the liver after exclusion of pancreatic juice from the intestine, the following possibilities may be considered:

1. A substance is excreted by the pancreas into the intestine that is necessary for the prevention of fatty livers.

2. The pancreas contains an internal secretion (in addition to insulin) that prevents fatty livers, and its production is destroyed either by the pressure resulting from the ligation of the ducts or by the associated atrophy of the gland. The experimental data offered by Dragstedt and his coworkers (5) in this connection have been dealt with above and need no further mention here.

3. The pancreatic juice or raw glandular tissue is only indirectly involved by making possible the absorption of some dietary constituent which inhibits the deposition of abnormal amounts of fat in the liver. Two factors may be referred to, protein and choline. It is well known that the digestion and absorption of proteins are interfered with when the pancreatic juice is excluded from the intestine. Channon and Wilkinson (21) and Best and Channon (22) have shown that a low protein diet will produce fatty livers in rats. It was first shown by Best *et al.* (4) and confirmed by Kaplan and Chaikoff (3) that choline, when fed as such, prevents and cures the fattiness of the liver of the completely depancreatized dog maintained with insulin; although the inactivity of the large amounts of choline present in meat was noted, it was nevertheless suggested that in the absence of pancreatic enzymes the choline combined in the lecithin of the meat is not so readily available to the animal as free choline.

SUMMARY

The effects of complete occlusion of the pancreatic ducts on liver lipids were investigated.

1. Fatty livers were observed in dogs examined at intervals of 12 to 24 weeks after complete disconnection of pancreatic ducts and intestine.

2. Fatty livers were observed in duct-ligated dogs fed a diet containing no raw pancreas regardless of whether they showed a marked loss, a slight loss, or no loss in body weight.

3. The addition of raw pancreas to the diet after duct ligation prevented the abnormal deposition of lipids in the liver.

4. The mechanism of the production of these fatty livers is discussed.

BIBLIOGRAPHY

1. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **108**, 201 (1935).
2. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **119**, 435 (1937).
3. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **120**, 647 (1937).
4. Best, C. H., Ferguson, G. C., and Hershey, J. M., *J. Physiol.*, **79**, 94 (1933).
5. Dragstedt, L. R., Van Prohaska, J., and Harms, H. P., *Am. J. Physiol.*, **117**, 175 (1936).
6. Chaikoff, I. L., and Kaplan, A., *J. Nutrition*, **14**, 459 (1937).
7. Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, **119**, 423 (1937).
8. Best, C. H., and Ridout, J. H., *Am. J. Physiol.*, **122**, 67 (1938).
9. Aylward, F. X., and Holt, L. E., Jr., *J. Biol. Chem.*, **121**, 61 (1937).
10. Channon, H. J., Loach, J. V., and Tristram, G. R., *Biochem. J.*, **32**, 1332 (1938).
11. Aubertin, E., Lacoste, A., and Castagnou, R., *Compt. rend. Soc. biol.*, **118**, 149 (1935).
12. Ralli, E. P., Rubin, S. H., and Present, C. H., *Am. J. Physiol.*, **122**, 43 (1938).
13. Person, E. C., and Glenn, F., *Proc. Soc. Exp. Biol. and Med.*, **40**, 56 (1939).
14. Van Prohaska, J., Dragstedt, L. R., and Harms, H. P., *Am. J. Physiol.*, **117**, 166 (1936).
15. Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, **115**, 557 (1936).
16. Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).
17. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
18. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
19. Macleod, J. J. R., Carbohydrate metabolism and insulin, London and New York, 20 (1926).
20. Allen, F. M., Studies concerning glycosuria and diabetes, Boston, 464 (1913).
21. Channon, H. J., and Wilkinson, H., *Biochem. J.*, **29**, 350 (1935).
22. Best, C. H., and Channon, H. J., *Biochem. J.*, **29**, 2651 (1935).

METHIONINE STUDIES

II. *dl*-METHIONINE SULFOXIDE*

By GERRIT TOENNIES and JOSEPH J. KOLB

(*From the Lankenau Hospital Research Institute, Philadelphia*)

(Received for publication, February 17, 1939)

In the light of preceding studies on the oxygenation of sulfur in the biologically occurring disulfide cystine and the mercaptan cysteine (1, 2) the behavior of the sulfide methionine toward peroxidic oxygen appeared of definite interest. In this connection we have recently reported the isolation of *dl*-methionine sulfoxide (3). It is the purpose of the present paper to communicate a simple method for the preparation of this compound and to render a more detailed account of some of its properties.

EXPERIMENTAL

Preparation—A simpler and more satisfactory method than that reported earlier (3) is as follows: Dissolve 200 mM of *dl*-methionine by means of concentrated hydrochloric acid (about 220 mM, 10 per cent excess), 150 cc. of water, and 250 cc. of methanol. Add 240 mM (about 20 per cent excess) of hydrogen peroxide in the form of its 30 per cent solution and some water to make the volume about 500 cc. (this is done in a volumetric flask if it is desired to follow the oxidation by titration). After the well mixed solution, which will require some cooling under the tap in the beginning, has been allowed to stand for 20 to 30 minutes, add about 230 mM (5 per cent excess) of amylamine (Sharples Solvents Corporation) and methyl alcohol equal to about one-half of the total volume of the reaction mixture. If a product free of dust or other insoluble particles is desired, filter the solution at this point through a Jena fritted glass filter (porosity No. 3). Add 3 volumes of acetone (about 2400 cc.) which

---

* Aided by a grant from the Blanche and Frank Wolf Foundation, Inc.

will precipitate the sulfoxide. The precipitate may be filtered off as soon as the supernatant liquid becomes clear (about 10 minutes). If left standing for a considerable period the precipitate is much more difficult to filter and some discoloration may be present. About six resuspensions in 500 cc. portions of acetone produce a negative chloride test, indicating substantial absence of amylamine hydrochloride. The white, powdered substance is dried by being left overnight in the air and finally heated for 1 hour at 100°. The yield is 95 per cent of the calculated amount.

General Properties—Titration of basic groups by the perchloric-acetic acid method (4) gave[1] a mean equivalent weight of 165.1, while titration of acid groups by the thymol blue-sodium methylate method (5) gave[2] values of 164.9 and 165.3. The calculated acid or basic equivalent weight is 165.2. Different preparations showed the same decomposition range of about 225–230°. 20 hours heating at 102° left the substance unchanged as far as appearance, per-oxygen content (*cf.* "Reduction"), and odor[3] are concerned. Likewise, an aqueous solution, after several weeks standing, showed no evidence of chemical changes, according to per-oxygen content and pH.

The solubility of the compound in water is very high; at about 25° it corresponds to 66 gm. per 100 gm. of water. The density of this solution is 1.157 and the molarity 2.80. The solubility exceeds that of most other α-amino acids and is about 12 times that of the (molar) solubility of the parent substance *dl*-methionine.

---

[1] The behavior at the end-points indicates a lower basicity than that of the ordinary neutral amino acids (4): on direct titration the yellow end-point is ill defined; the green end-point gave an equivalent weight of 164.8 and 165.3. Titration in presence of 1 cc. of formic acid and 20 cc. of acetic acid gave, for the mean value between the green and yellow end-points (0.10 cc. of 0.10 N $HClO_4$ difference), 165.0 and 165.1.

[2] About 1 mM was dissolved by 0.3 or 0.4 cc. of H_2O, and titrated, after addition of 2 drops of 0.003 M thymol blue-sodium salt in methanol, with 0.1 N $NaOCH_3$ in methanol to a blue end-point. The blank correction to be deducted was obtained by titrating to the same color after adding to the titrated solution water, indicator, and methanol in amounts equal to those already present.

[3] The characteristic "methionine" odor, which has been described as that of boiled cabbage, was noted on all specimens, even after several reprecipitations.

Reduction—The substance is not reduced, nor does it apparently otherwise interfere, in the cystine determination at pH 5 by Folin's reagent (6). Our preparations gave a disulfide test corresponding to a cystine content of 0.05 per cent, presumably owing to some impurity of the synthetic *dl*-methionine used which showed a similar value (0.03 per cent) and a sulfur content of 21.9 per cent (calculated 21.5). By hydriodic acid, under the conditions established by Lavine (7) for the complete reduction of cystine disulfoxide, the compound is reduced to an insignificant extent. However, by further increasing acidity and iodide concentration, conditions are reached where practically complete reduction prevails. This is shown by determinations in which 1 mM of the sulfoxide was allowed to react (in the dark), in a total volume of 10 cc., with different concentrations of freshly distilled hydriodic acid, while 1 or 2 cc. samples were periodically withdrawn and, after dilution with 10 to 20 volumes of water, titrated with 0.025 N thiosulfate. Blank values were obtained under identical conditions, except for omission of the sulfoxide. The fraction of the calculated amount of iodine found was, with 3.8 M HI, 11, 57, 70, 81, 92, 98 per cent, after 6, 30, 60, 100, 140, and 180 minutes respectively, but when 7.6 M HI (57 per cent) was used the percentages were 95, 97, 97 after 3, 24, and 48 minutes.

Attempts were made to circumvent the inconveniences attending the use of concentrated, hypophosphite-free, hydriodic acid. Combinations of hydrochloric or hydrobromic acid and soluble iodides of sufficiently high concentration cannot be used because iodine is not stable in such solutions (3.7 M LiI + 3.4 M HCl or HBr) owing to the formation of the iodine halides (ICl, IBr). Decolorization of iodine also takes place in sulfuric acid solutions of lithium iodide (3.7 M LiI and 5 M H_2SO_4), accompanied by formation of hydrogen sulfide, as well as in the presence of phosphoric acid (3.7 M LiI and 7.4 M H_3PO_4). The problem was solved by the use of perchloric acid. Iodine proved to be perfectly stable in perchloric acid solutions of sodium iodide. This salt was used because it is soluble to the extent of 8 M at room temperature and because the solubility of sodium perchlorate is much higher than that of lithium perchlorate, 8 M sodium iodide solution when stored in the dark develops little iodine color. Reduction in 2 M $HClO_4$ and 4 M NaI was found to be much slower

(34 per cent completion after 3 minutes, 97 per cent after 100 minutes) than in 4 M $HClO_4$ and 4 M NaI (97 per cent after 2 minutes), while in 4 M $HClO_4$ and 2 M NaI the maximum value is obtained after 30 to 60 minutes. The latter concentrations (with 0.1 M sulfoxide) were adopted as being most convenient practically. An experiment made for the purpose of testing the precision and accuracy of the method is reproduced in Table I. The 5-fold increase in the ratio HI to sulfoxide which forms part of this experiment leaves the result substantially unchanged, suggesting that the reaction is complete and the blank correction valid. The product used, which was obtained as described above without further purification, thus may be considered 99.8 to 99.9 per cent pure.

TABLE I

Reduction of dl-Methionine Sulfoxide in Sodium Iodide-Perchloric Acid Solution

All three solutions were kept in the dark except when samples were drawn. Samples were pipetted into 5 volumes of water (10 cc. and 50 cc. respectively) and titrated with 0.02484 N $Na_2S_2O_3$ which had been standardized against iodate as well as against copper. The burette was calibrated, while pipettes and volumetric flasks were used without corrections. In calculating the results one-fifth of the blank values of Column 4 were subtracted from the values of Column 2, and the full values of Column 4 from those of Column 3. The thus corrected mean value of Column 2 is 99.91 ± 0.03 per cent, and that of Column 3, 99.76 ± 0.06 per cent of the theoretical.

Time (1)	Iodine liberated by 165.0 mg. methionine sulfoxide in 10 cc. 2.0 M NaI + 4.1 M $HClO_4$, per 2 cc. sample (2)	165.3 mg. methionine sulfoxide in 50 cc. 2.0 M NaI + 4.1 M $HClO_4$, per 10 cc. sample (3)	No methionine sulfoxide, 50 cc. 2.0 M NaI + 4.1 M $HClO_4$, per 10 cc. sample (4)
min.	*cc.* $Na_2S_2O_3$	*cc.* $Na_2S_2O_3$	*cc.* $Na_2S_2O_3$
60	16.12	16.30	0.22
100	16.14	16.40	0.34
140	16.16	16.45	0.36
180		16.56	0.48

It seemed of interest to establish the behavior of methionine sulfoxide with cysteine. The two compounds (*l*-cysteine (8) was used) were combined in the ratio corresponding to the reaction 2 cysteine + 1 methionine sulfoxide = 1 cystine + 1 methionine + 1 H_2O, and the progress of interaction was observed polarimetrically. The results, in water and in 2 M $HClO_4$, are reproduced in Fig. 1. They show a slow reaction, somewhat faster in acid than in neutral solution, the rate of which is consistent with

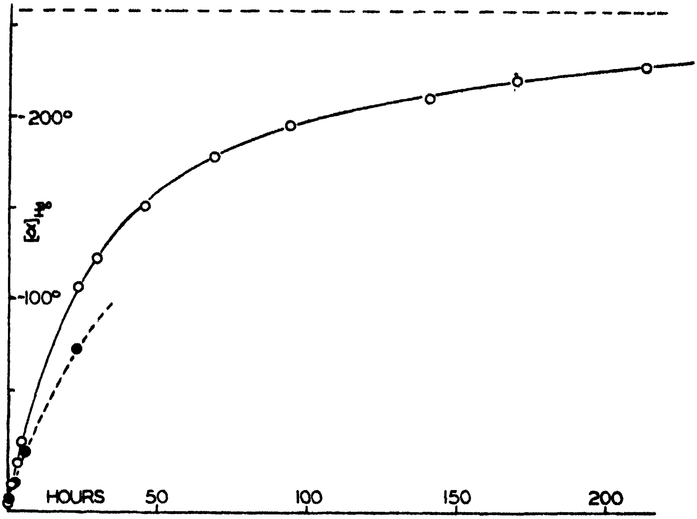


FIG. 1. The reaction between *dl*-methionine sulfoxide and *l*-cysteine. ○ 0.25 M methionine sulfoxide and 0.50 M cysteine in 2 M $HClO_4$. The specific rotation of *l*-cystine (0.25 M) dissolved with *dl*-methionine (0.25 M) in 2 M $HClO_4$, according to the presumed final state in this reaction, was in a separate experiment found to be $[\alpha] = -258°$. This value is indicated by the dotted horizontal line. ● 0.21 M methionine sulfoxide and 0.42 M cysteine in H_2O. In this series four separate experiments (6 cc. in 10 cc. volumetric flasks) were started simultaneously; cystine precipitation was in evidence after 2 hours; optical readings were obtained by adding, to each sample at the time shown in the graph, perchloric acid to make the concentration 2 M, and water to make the total volume 10 cc. Temperature 25–28°. The ordinate represents specific rotations, calculated for cystine.

the assumption of a bimolecular process as the rate-controlling step.

$$\text{MSO} + \text{RSH} \rightarrow \text{MS} + \text{RSOH (slow)}$$
$$\text{RSOH} + \text{RSH} \rightarrow \text{RSSR} + H_2O \text{ (fast)}$$

The slowness of this reaction of the monosulfoxide of methionine contrasts with the instantaneous reaction of cystine disulfoxide (7) which is related to the instability of the —S—S— linkage.

Salts—The aqueous solution gives no precipitate with mercuric chloride, neither directly, as does methionine, nor on neutralization. With cupric acetate, which in a pure methionine solution causes immediate crystallization of the copper salt, the sulfoxide gives a deep blue color, but no insoluble product. A characteristic derivative of the sulfoxide is the picrate, obtained by dissolving equimolar amounts of the two components in a small amount of hot water. On cooling a fine yellow precipitate crystallizes.

	mol. wt.
$C_5H_{11}O_3NS \cdot C_6H_3O_7N_3$	394.2
Found acidimetrically (brom-cresol green, NaOH)	397.5
" iodometrically (*cf.* "Reduction")	388

Methionine gives no crystalline salt under similar conditions, two oily layers resulting.

DISCUSSION

Enhancement of hydrophilic tendencies and acidic properties by oxygenation of sulfur was evident in the increased (20 times) solubility and lower isoelectric point of cystine disulfoxide (1) compared with cystine. It is equally evident in the comparison of methionine and methionine sulfoxide (30 times increased solubility; acidic properties in perchloric acid titration in acetic acid). The oxygen potential of the compound, to judge from the behavior with iodide and with cysteine, is much lower than that of the cystine derivative. Possibly related hereto is the absence of any tendency to form sulfenic or sulfinic acids by hydrolytic cleavage, which is so pronounced in the case of cystine disulfoxide and characteristic of the —S—S— linkage.

The present product has an interesting stereochemical aspect. Oxidation to the sulfoxide renders the sulfur atom asymmetric. Hence the product of this reaction may be a mixture of four optical isomers present as two racemic modifications. In connection with an examination of the possible metabolic significance of the sulfoxide separation of the four stereoisomers should be of interest.

SUMMARY

A method is given for the conversion of *dl*-methionine into a sulfoxide, by oxidation with hydrogen peroxide in acid solution. Some properties and reactions of the new compound are shown, including its quantitative reduction by hydriodic acid and its reaction with cysteine.

BIBLIOGRAPHY

1. Toennies, G., and Lavine, T. F., *J. Biol. Chem.*, **113**, 571 (1936).
2. Toennies, G., *J. Biol. Chem.*, **122**, 27 (1937–38).
3. Toennies, G., *Science*, **88**, 545 (1938).
4. Toennies, G., and Callan, T. P., *J. Biol. Chem.*, **125**, 259 (1938).
5. Lavine, T. F., and Toennies, G., *J. Biol. Chem.*, **101**, 727 (1933).
6. Shinohara, K., *J. Biol. Chem.*, **112**, 683 (1935–36).
7. Lavine, T. F., *J. Biol. Chem.*, **113**, 583 (1936).
8. Toennies, G., and Bennett, M. A., *J. Biol. Chem.*, **112**, 497 (1935–36).

STUDIES ON THE EFFECTS OF CALCIFEROL IN THE THYROPARATHYROIDECTOMIZED-NEPHRECTOMIZED RAT*

BY WILBUR R. TWEEDY, R. D. TEMPLETON, MARY C. PATRAS, F. A. McJUNKIN, AND E. W. McNAMARA

(From the Departments of Biological Chemistry, Physiology, and Pathology, Loyola University School of Medicine, Chicago)

(Received for publication, February 10, 1939)

Various views are held regarding the manner in which massive doses of irradiated ergosterol, or other antirachitic preparations, produce an elevation in the blood calcium. The most plausible among these views appear to be the following: (1) The antirachitic agent stimulates the parathyroids to great activity. (2) The antirachitic vitamin influences the net absorption[1] of calcium from the intestinal tract and, therefore, affects the serum calcium level. (3) The antirachitic agent acts directly on the organic matrix of the bone, releasing calcium to the blood. (4) A toxic by-product of the irradiation of the sterol is responsible for the hypercalcemia which often results after the injection of massive doses of the antirachitic preparation.

In 1928 Hess and Lewis (2) offered evidence in support of the view, first advanced by Block and Faber (3), that irradiated ergosterol acts through stimulation of the parathyroid glands. Hess and Lewis stated that the hypocalcemia induced in dogs and monkeys by feeding diets low in calcium could be converted into hypercalcemia by means of irradiated ergosterol only when the parathyroids are intact, and not in their absence. This view was later abandoned when it was found by Hess, Weinstock, and Rivkin (4) that hypercalcemia could be produced in the parathyroidectomized animal if the dose of irradiated ergosterol was sufficiently great. Jones (5), Wade (6), Shelling (7), Comel (8), Urechia and Popoviciu (9), Demole and Christ (10), Brougher (11), and Reed and Seed (12) have presented evidence which may be interpreted as supporting the latter view.

---

* Presented before the American Society of Biological Chemists at Memphis, April, 1937, and before the Sixteenth International Physiological Congress at Zurich, August, 1938.

[1] Harris and Innes (1) define the term as follows: "*Net absorption* = Ca (or P) intake *minus* fæcal output = absorption from gut *minus* excretion into gut."

Taylor and his associates (13, 14) are of the opinion that due attention has not been accorded the possible presence of parathyroid "rests," which may have been stimulated by the large doses of antirachitic agent used by many of the above workers. In support of their view they submitted their observations on two groups of dogs, one of which had been subjected to thyroparathyroidectomy, and the other to thyroparathyroidectomy and careful resection of all aberrant neck tissue. In the first group the vitamin D preparation alleviated the symptoms of parathyroid tetany within 1 hour after administration, but in the second group it was without effect. Dale, Marble, and Marks (15) have failed to obtain full confirmation of the experiments of the above workers. They found that their dogs developed a high level of calcium in the blood on the administration of the antirachitic agent as readily after removal of the parathyroids as before the operation. Workers in our laboratories (16, 17) have submitted evidence which minimizes the importance of accessory parathyroid tissue in the rat. They are of the opinion that various controllable factors, such as temperature, immediate dietary treatment, etc., are far more important than accessory parathyroids in preventing tetany in the rat.

Harris and Innes (1) contend that irradiated ergosterol tends to increase the absorption of calcium and phosphorus, or to diminish the "back absorption" of these substances into the gut. They believe that their experimental results are not inconsistent with the simple view that vitamin D acts by increasing the apparent solubility of Ca and phosphate in the blood.

Shelling (7) is of the opinion that the antirachitic agent acts directly on the organic matrix of the bone, releasing calcium to the blood. He has shown that hypercalcemia and calcification in certain vital organs can be produced by the oral administration of viosterol in parathyroidectomized rats which are on a calcium-free diet.

Tweedy and McNamara (18) have shown in the nephrectomized rat in which the parathyroids are intact that the serum calcium drops very gradually during the remaining life span (48 to 72 hours) of the animal, even though the serum inorganic phosphorus may reach 20 mg. per cent during the same interval (19). McJunkin, Tweedy, and McNamara (20) observed that in the above animal massive doses of calciferol produce hypercalcemia, if the injections are begun within 5 hours after nephrectomy when the serum calcium-phosphorus ratio is not greatly changed. They also observed slight to marked osteoclastic resorption in the femurs. If the injections were delayed longer than 5 hours after nephrectomy, bone resorption was sometimes observed, but hypercalcemia, if it occurred, was not present at the end of 48 hours. After the delayed injections, however, the serum calcium did not fall but increased slightly.

In the present work it will be shown that if the thyroparathyroidectomized rat is allowed to develop a low serum calcium-phosphorus ratio and is then nephrectomized, the subsequent injection of calciferol produces no apparent effects. Hypercalcemia is not produced, nor are there areas of resorption in the long bones. Thus removal of the kidneys appears to render the thyroparathyroidectomized rat immune to the action of calciferol, or to any toxic impurity that may be present in the antirachitic preparation. If, however, the serum calcium-phosphorus ratio of the thyroparathyroidectomized rat is converted into a normal value by the administration of a high calcium-low phosphorus diet, then nephrectomy does not prevent the production of hypercalcemia by calciferol.

Materials and Methods

Albino rats of the Wistar strain were used. These animals ranged from 60 to 120 days in age, and from the time of weaning were maintained on a diet, which consisted of Fox Chow (a commercial diet), supplemented by meat scraps, and cabbage leaf twice weekly. The Fox Chow contained about 1.36 per cent of calcium, and 0.98 per cent of phosphorus. The rachitogenic diet used was the Jones modification (21) of the Steenbock-Black rachitogenic Ration 2965 (22). This diet consisted of commercial yellow corn-meal 66 parts, wheat gluten 20 parts, wheat embryo 10 parts, calcium carbonate 3 parts, and sodium chloride 1 part. Food was withdrawn after the animals were bilaterally nephrectomized, but they were allowed water during the postoperative period. In each case the animal was bedded on cotton, and kept at a temperature which did not vary greatly from 24°. Blood samples were taken by cardiac puncture, when the animals usually showed no more than a moderate degree of toxicity as judged by their activity. Serum calcium determinations were made by the Kramer-Tisdall method as modified by Tweedy and Koch (23), and serum inorganic phosphorus by Bodansky's method (24).

Recognition of bone resorption was not attempted except in connection with osteoclastic activity. A description of the histological technique employed is given in a previous paper (20).

Crystalline calciferol (40,000 I.U. per mg.) was dissolved in fresh Mazola oil just before being used, and injections were made, subcutaneously, with a 1 cc. tuberculin syringe.

EXPERIMENTAL

Fifteen rats (Nos. 1 to 15, Table I), which were thyroparathyroidectomized, had an average serum calcium of 6.49 mg. per cent 3 to 5 days after the operation, and in several other animals the serum inorganic phosphorus ranged from 8 to 10 mg. per cent. After being on the stock diet for about 1 month, these animals were removed from the diet and immediately bilaterally nephrectomized. The first injection of calciferol was made within 1 hour after nephrectomy, and the next two at 6 and 18 hours, respectively. In Table I are shown the individual serum calcium values 48 hours after nephrectomy. It will be seen that the serum calcium level in thirteen of the fifteen animals appears to have been unaffected by the calciferol.

Seventeen rats which had been placed on a low calcium diet immediately after thyroparathyroidectomy exhibited definite signs of tetany within 48 hours. These animals were placed on the Jones-Steenbock rachitogenic diet and remained on it for approximately 1 month. After this period of dietary treatment the serum calcium in nine of the animals (Group II, Table I) varied from 7.6 to 10.2 mg. per cent, with an average value of 9 mg. per cent, and the serum inorganic phosphorus in four of these animals ranged from 4 to 5 mg. per cent. Five of these animals, and the remaining eight animals from which blood samples had not been taken, were continued on the rachitogenic diet for 10 days. These thirteen animals were then removed from the diet and immediately nephrectomized. Eight were injected with calciferol, and five were not injected. 48 hours after nephrectomy blood samples were collected and analyzed

TABLE I

Effect of Calciferol (Vitamin D_2) on Serum Calcium of Thyroparathyroidectomized Rats after Bilateral Nephrectomy

Group I, stock diet			Group II, rachitogenic diet					
Rat No.	Serum Ca		Litter	Rat No.	Serum Ca, controls		Bilaterally nephrectomized + vitamin D_2†	
	Before nephrectomy	After nephrectomy + vitamin D_2*			Before nephrectomy	After nephrectomy	Serum Ca	Serum inorganic P
	mg. per cent	*mg. per cent*			*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	6.05	6.73	A	16			12.00	7.03
2	6.42	6.54		17		9.00		
3	6.79	6.08		18			19.00	
4	6.42	5.07		19	9.00			
5	5.87	9.41	B	20		7.80		
6	6.60	8.25		21			13.00	
7	7.51	6.46		22	8.88			
8	6.42	6.87	C	23	7.60		12.40	11.64
9	6.05	10.75						
10	7.15	5.11	D	24		9.40		
11	6.60	6.70		25	8.00		10.05	
12	6.64	7.09	E	26		9.20		
13	6.28	5.45		27	9.00		14.60	12.30
14	5.02	5.36		28	10.20			
15	7.53	6.09	F	29	9.50		11.30	8.40
				30		8.00		
				31	9.40		12.20	10.30
				32	9.40			
Average....	6.49	6.80			9.00	8.68	13.07	9.93

* 3 to 6 × 460,000 I.U.

† 3 × 460,000 I.U.

for calcium and phosphorus. In the animals injected with calciferol, the serum calcium varied from 10 to 19 mg. per cent, with an average value of 13 mg. per cent, and the serum inorganic phosphorus averaged 9.93 mg. per cent. The difference of 4 mg. per cent in the serum calcium of controls and injected animals shows that the calciferol which had been injected was effective in causing a mobilization of calcium into the blood.

In view of our observation that osteoclast activity was absent from the femurs of the injected animals, it appeared that the calcium mobilized by the calciferol might be drawn from the soft tissues, the food in the gastrointestinal tract, or from both the food and the soft tissues. The following experiment was then carried out. Eight rats were thyroparathyroidectomized and kept on the Jones-Steenbock rachitogenic diet for 30 days. 24 hours before they were nephrectomized, four of these animals were placed on a diet that was low in all mineral constituents in order to sweep excess calcium from the gastrointestinal tract. The other four animals were continued on the high calcium-low phosphorus diet until they were nephrectomized. Then three of the latter group were injected with calciferol in the same amount and manner used for the rats in Group II, Table I. At the end of 48 hours these three animals showed serum calcium values of 12.8, 13.4, and 14.7 mg. per cent, respectively. The uninjected animal showed a serum calcium value of 8.28 mg. per cent. In the four animals which had been changed to the low mineral diet, nephrectomized, and injected with calciferol as described above, we found the serum calcium to be 8.0, 8.1, 8.2, and 13.8 mg. per cent, respectively. These data clearly indicate that where calcium was present in the gastrointestinal tract of the animal it was absorbed, and accumulated in the blood if calciferol was injected.

DISCUSSION

It is a well established fact that rats which are maintained on a diet that is optimal for bone growth usually show a drop in serum calcium and a rise in serum inorganic phosphorus within 24 to 48 hours after parathyroidectomy. In the present experiments we found an average serum calcium of 6.49 mg. per cent, 3 to 5 days after thyroparathyroidectomy (Rats 1 to 15, Table I), and in previous work (25) we have shown that this low level in in the serum calcium may be present for 6 or 7 months before a return to low normal values is indicated. In several other animals, similarly treated, we found that the serum inorganic phosphorus is regularly 8 to 10 mg. per cent when the above level in serum calcium is reached.

Shelling (26) has shown that massive doses of viosterol greatly increase the excretion of calcium and phosphorus in normal rats, and that viosterol directs the excretion of the extra calcium and phosphorus away from the bowel towards the kidneys. In Rats 1 to 15 (Table I), the serum calcium was low, and the serum inorganic phosphorus was presumably high at the time the injections of calciferol were begun. Consequently it would appear that the amount of calcium entering the blood was greatly restricted by the more rapid accumulation of inorganic phosphorus in the blood. The rise in serum calcium above the control values in Rats 5 and 9 (Table I) may have been due to the calciferol injected, or it may have occurred during the interval between thyroparathyroidectomy and nephrectomy. However, the absence of any evidence of osteoclast activity in the bones and the low serum calcium in thirteen of the fifteen animals clearly indicate that calciferol had little effect in mobilizing calcium into the blood.

In the animals of Group II (Rats 16 to 32, Table I), in which there was presumably a normal serum calcium-phosphorus ratio at the time of nephrectomy, the injection of calciferol caused an increase in the serum calcium of all the animals that were injected, and hypercalcemia in two of them. The fact that the bones of these animals showed the absence of more than the normal number of osteoclasts indicates that the primary action of calciferol was elsewhere. Furthermore, the failure of calciferol to produce an increase in the serum calcium of three of the four thyroparathyroidectomized rats from which the high calcium-low phosphorus diet was withdrawn 24 hours before they were nephrectomized, and the positive action of calciferol in the three animals left on the diet until nephrectomy, strongly suggest that the source of the extra calcium was the gastrointestinal tract.

Watchorn (27), Brown and Shohl (28), and Shelling (26) have shown that irradiated ergosterol decreases calcium excretion in the feces and increases urinary excretion of calcium. Presumably in our thyroparathyroidectomized rats (Group II, Table I) that were nephrectomized and injected with calciferol, the accumulation of calcium in the blood was due to the action of calciferol in greatly restricting the fecal excretion of calcium. Since at the same time the fecal excretion of phosphorus was probably greatly decreased, its accumulation in the blood served to decrease, or to limit the absorption of calcium. The failure of the bones of these animals to undergo resorption may have been due to the retention of calcium to a greater extent than that which occurred in our nephrectomized rats in which the parathyroids were intact (20), and in which we found both bone resorption and hypercalcemia after the injection of calciferol. The present experiments strongly suggest that massive doses of calciferol act by decreasing the fecal excretion of calcium.

It would seem that our experiments support the view that the toxic action of calciferol arises through its effects on the calcium and phosphorus metabolism. Furthermore, it is suggested that the necrotic lesions of parenchymal tissues that often follow the injection of excessive amounts of calciferol are secondary to disturbances in the calcium and phosphorus metabolism.

SUMMARY

Administration of massive doses of calciferol to thyroparathyroidectomized-nephrectomized rats, in which there is a low serum calcium-inorganic phosphorus ratio, is ineffective in producing hypercalcemia. If, however, appropriate dietary treatment is instituted soon after thyroparathyroidectomy so that the normal relationship of calcium to phosphorus in the blood is restored, or maintained, then calciferol is effective in promoting mobilization of calcium into the blood of such animals after they have been nephrectomized.

The calciferol used in these experiments was generously supplied by Mead Johnson and Company through the courtesy of Dr. C. E. Bills.

BIBLIOGRAPHY

1. Harris, L. J., and Innes, J. R. M., *Biochem. J.*, **25**, 367 (1931).
2. Hess, A. F., and Lewis, J. M., *J. Am. Med. Assn.*, **91**, 783 (1928).
3. Block, C. E., and Faber, F., *Am. J. Dis. Child.*, **30**, 504 (1925).
4. Hess, A. F., Weinstock, M., and Rivkin, H., *Proc. Soc. Exp. Biol. and Med.*, **27**, 298 (1929–30).
5. Jones, J. H., *J. Biol. Chem.*, **70**, 647 (1926).
6. Wade, P. A., *Am. J. Med. Sc.*, **177**, 790 (1929).
7. Shelling, D. H., *J. Biol. Chem.*, **96**, 215 (1932).
8. Comel, M., *Atti accad. Lincei*, **11**, 857 (1930); *Arch. fisiol.*, **29**, 123 (1930).
9. Urechia, C. I., and Popoviciu, G., *Compt. rend. Soc. biol.*, **98**, 405 (1928).
10. Demole, V., and Christ, A., *Arch. exp. Path. u. Pharmakol.*, **146**, 361 (1929).
11. Brougher, J. C., *Am. J. Physiol.*, **84**, 583 (1928); *Clin. Med. and Surg.*, **38**, 167 (1931).
12. Reed, C. I., and Seed, L., *Endocrinology*, **17**, 136 (1933).
13. Taylor, N. B., *J. Am. Med. Assn.*, **96**, 60 (1931).
14. Taylor, N. B., Weld, C. B., Brannon, H. D., and Kay, H. D., *Canad. Med. Assn. J.*, **24**, 763 (1931).
15. Dale, H., Marble, A., and Marks, H. P., *Proc. Roy. Soc. London, Series B*, **111**, 522 (1932).
16. Patras, M. C., Galapeaux, E. A., and Templeton, R. D., *Am. J. Physiol.*, **122**, 409 (1938).
17. Templeton, R. D., Borkon, E. L., Genitis, V., and Galapeaux, E. A., *Endocrinology*, **21**, 541 (1937).
18. Tweedy, W. R., and McNamara, E. W., *Proc. Soc. Exp. Biol. and Med.*, **35**, 414 (1936).
19. McJunkin, F. A., Tweedy, W. R., and Mencky, W. J., *Arch. Path.*, **18**, 626 (1934).
20. McJunkin, F. A., Tweedy, W. R., and McNamara, E. W., *Am. J. Path.*, **13**, 325 (1937).
21. Jones, J. H., *J. Biol. Chem.*, **106**, 701 (1934).
22. Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).
23. Tweedy, W. R., and Koch, F. C., *J. Lab. and Clin. Med.*, **14**, 747 (1929).
24. Bodansky, A., *J. Biol. Chem.*, **99**, 197 (1932–33).
25. Tweedy, W. R., and Chandler, S. B., *Am. J. Physiol.*, **88**, 754 (1929).
26. Shelling, D. H., *J. Biol. Chem.*, **96**, 229 (1932).
27. Watchorn, E., *Biochem. J.*, **24**, 631 (1930).
28. Brown, H. B., and Shohl, A. T., *J. Biol. Chem.*, **86**, 245 (1930).

THE DETERMINATION OF HYDROGEN PEROXIDE IN BACTERIAL CULTURES

By EDNA R. MAIN and LAWRANCE E. SHINN

(From the Institute of Pathology, the Western Pennsylvania Hospital, Pittsburgh)

(Received for publication, February 4, 1939)

The accumulation of hydrogen peroxide in bacterial cultures may contribute to the bacteriostatic effect exerted by sulfanilamide (1) on organisms which produce peroxide and are sensitive to its action. The methods commonly used for determination of small quantities of peroxide are not suitable for analysis of broth cultures because of the color of the mixture and the presence of reducing substances. In view of the increasing importance of sulfanilamide and related compounds as chemotherapeutic agents and the general interest in the rôle of peroxide in bacterial metabolism, the need for a method applicable to cultures is apparent. The method presented here is an adaptation of the commonly used qualitative method (2) in which hydrogen peroxide is detected by the oxidation of benzidine or tolidine in the presence of peroxidase. By substituting an extract for the small piece of potato usually used, the entire solution acquires the blue-green color and colorimetric comparisons with standards can be made. The method gave results which were entirely satisfactory for the purpose for which it was designed and should be useful in similar problems.

Method

Reagents—

1. A 10 per cent solution of *o*-tolidine in glacial acetic acid.

2. Potato extract. Allow 120 gm. of freshly ground potato to stand overnight in the ice box in about 130 cc. of a solution containing equal volumes of glycerol and phosphate buffer, pH 7.2. The mixture should be covered with a layer of mineral oil during the period of extraction and during subsequent use. After de-

canting and slight centrifugation to remove the coarser sediment, the extract is ready for use and is conveniently kept in a 50 cc. burette, protected by a layer of oil about 5 cm. in depth. The extracts usually retain their peroxidase activity for several days.[1] Each extract is standardized on the day it is used with known amounts of peroxide as described below.

3. A 1 per cent aqueous solution of malachite green oxalate (National Aniline and Chemical Company) serves as a stock solution from which dilutions are made for the preparation of permanent standards. A series of fifteen dilutions ranging in concentration from 0.00015 to 0.02 per cent is adequate for the comparison with the concentrations of peroxide found in the cultures tested. When sealed in small tubes, the diluted solutions can be kept indefinitely.

4. A 30 per cent solution of hydrogen peroxide (Merck's Reagent) to be used for calibrating the permanent standards.

Procedure of Analysis—Samples of 1.0 cc. are placed in small tubes of equal diameter. To each is added 6 drops of potato extract and, immediately after, 2 drops of *o*-tolidine reagent. After the period required for the development of maximal color intensity (see section below), comparisons are made with appropriate standards in a comparator block. A blank, containing only sterile broth, potato extract, and *o*-tolidine, is prepared for each set of determinations and is placed back of the standard solution to compensate for the turbidity. No color can be detected in the blanks. The peroxide content is calculated by reference to calibration curves.

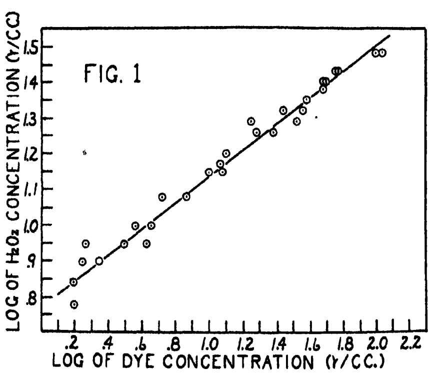
Calibration of Standards—Broth dilutions, containing from 6 to 30 micrograms of peroxide per cc. are prepared from the 30 per cent reagent.[2] The first dilutions, to 0.06 and 0.03 per cent, are prepared in water to avoid possible destruction by catalase, which is present in appreciable amounts in some lots of broth. Broth used for the dilutions is the same as that used as the culture medium. The required amounts of the water dilutions are measured with 0.1 cc. pipettes, graduated in hundredths, into the bottoms of the tubes and the total volume is made up to 1 cc. with broth. To each tube are added 6 drops of potato extract and 2 drops of *o*-tolidine reagent. The colors are compared with standards in a comparator block, with a blank as described above. The logarithms of the dye concentrations plotted against the logarithms of the corresponding peroxide concentrations have a linear relationship, as shown in Fig. 1.

---

[1] A flocculent precipitate which frequently forms on standing is allowed to settle and may be drawn off without affecting the activity of the extract.

[2] The reagent used contained 30.6 per cent hydrogen peroxide, determined by permanganate titration. The dilutions used in preparation of the standard curves were made immediately before use.

The peroxide content of the sample analyzed can be determined by reference to this curve. For practical purposes a more simply constructed curve has been found useful. The concentrations of dye found to be most suitable are 0.00015, 0.0003, 0.00045, 0.0006, 0.0009, 0.0012, 0.0015, 0.0018, 0.0025, 0.0037, 0.005, 0.0075, 0.01, 0.015, and 0.02 per cent. By assigning small, arbitrary numbers equally spaced, as 1, 1.5, 2, 2.5 . . . 8, to each of the above concentrations, the estimation of intermediate intensities is facilitated. The known concentrations of peroxide employed (6, 9, 12, 15, 18, and 24 micrograms per cc.) are plotted against the arbitrary numbers of the dye standards of equivalent intensity. Examples of curves of this type are illustrated in Fig. 2. The wider spread in the lower concentrations makes this method of plotting advantageous.



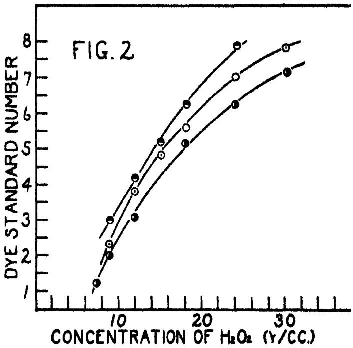


Fig. 1. Linearity of the relationship between logarithms of concentrations of H_2O_2 producing the coloration measured and logarithms of concentrations of malachite green oxalate producing a comparable intensity of color.

Fig. 2. Representative calibration curves as plotted for daily use.

Calibration curves should be made on the same day as the analyses, since the activity of an extract decreases from day to day.

Period of Maximal Color Development—When freshly prepared potato extracts are used, the color develops to its maximal intensity within 4 to 6 minutes. The time to be used for each extract is determined by making comparisons with known amounts of peroxide over periods of about 8 minutes. Fig. 3 shows the development of color with time for four concentrations of peroxide in broth. In pneumococcus cultures of comparable peroxide content, maximal color is developed in substantially the same time. As may be seen from the curves, the maximal color persists for a time sufficiently long for comparisons to be made before fading begins. Since the time used in the determination of unknowns is the same as that used in the calibration, differences of 1 to 2 minutes in the time selected will introduce no appreciable error.

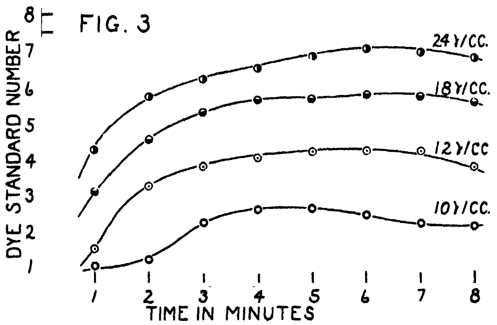


Fig. 3. Curves showing color development with time in various H_2O_2 concentrations.

Sensitivity and Accuracy

With the technique described the method is satisfactorily used in the range of 6 to 30 micrograms per cc.[3] The lower detectable limit of concentration varies from 4 to 8 micrograms per cc. and depends on the activity of the potato extract used. Colors developed by concentrations of less than 6 micrograms per cc. are usually too faint for accurate comparison. For concentrations greater than 30 micrograms per cc., it is necessary to make an appropriate dilution of the sample before the reagents are added. Dilutions made after the color is developed give incorrect results.

---

[3] Equal to 0.006 to 0.03 mg. per cc. or 6 to 30 parts per million.

Dilutions of hydrogen peroxide prepared as for standardization of dye solutions were analyzed as unknowns. The results are given in Table I. The distribution of errors in the determinations is uniform with respect to sign and magnitude so that, in a sufficiently large number, the average concentration found approaches closely the theoretical value. The frequency of the occurrence of determinations with error exceeding 15 per cent is given by the last two columns of Table I.[4]

TABLE I

Determination of Hydrogen Peroxide in Broth

Samples of 1 cc. volume were treated with 6 drops of potato extract and 2 drops of *o*-tolidine reagent. The colors developed were compared with standard dye solutions after 5 to 6 minutes.

Concentration of H_2O_2 present	Average concentration of H_2O_2 found	No. of determinations made	No. of determinations found to be > 15 per cent in error
micrograms per cc.	*micrograms per cc.*		
24.0	23.6	6	1
18	17.7	8	1
15	15.8	6	0
12	12.0	10	0
9	8.9	10	1
6	5.9	7	2

Hydrogen peroxide, added to broth cultures of the pneumococcus, is recoverable within the limits of accuracy of the method, provided the time of contact is sufficiently short to avoid effects of the constantly changing conditions in the culture. The amount of peroxide present in bacterial cultures at a given time depends upon the rate of its decomposition by catalase as well as upon the rate of formation by the organisms. The pneumococcus cultures to which the method has been applied contain the minimum amounts of added catalase required for support of the desired growth. In cultures containing more catalase, 100 per cent recovery of added peroxide would not be expected.

---

[4] A contributing source of error may lie in the difficulty encountered in preparing and measuring dilutions of 30 per cent peroxide. A marked tendency to drop formation on the glass appeared consistently.

DISCUSSION

The various qualitative and quantitative micromethods, by which 1 microgram or less of hydrogen peroxide can be detected, can be used only in the absence of color or interfering organic compounds. The method presented here offers no advantages over titrimetric or spot test methods where these are applicable. Calibration curves and analyses with water as the diluent show no greater degree of sensitivity than is found in the broth dilutions.

TABLE II

Production of Hydrogen Peroxide in Broth Cultures of Type I Pneumococcus

Medium	Age of culture	Concentration of H_2O_2
	hrs.	*micrograms per cc.*
Veal infusion broth	12	5
	14	6
	16	9
	18	12
Same, containing 10 mg. % sulfanilamide	12	12.5
" 10 " % "	14	14
" 10 " % "	16	15.5
" 10 " % "	18	16

The usefulness of the method lies chiefly in its adaptability to bacterial cultures or similar preparations. An illustration of this use is given in Table II, which shows the accumulation of hydrogen peroxide in broth cultures of pneumococcus in the presence and absence of sulfanilamide.

The reagents and experimental conditions adopted are suitable for the particular type of medium employed. Under different conditions or for other kinds of media, adjustments in acidity and other factors may be necessary.

SUMMARY

A simple and rapid colorimetric method is presented for the determination of hydrogen peroxide in bacterial cultures. The

method involves the oxidation of *o*-tolidine by peroxide in the presence of a peroxidase extract. Concentrations in the range of 6 to 30 micrograms per cc. are determined with an accuracy of 85 per cent or more.

BIBLIOGRAPHY

1. Locke, A., Main, E. R., and Mellon, R. R., *Science*, **88**, 620 (1938); *J. Immunol.*, **36**, 183 (1939). Shinn, L. E., Main, E. R., and Mellon, R. R., *Proc. Soc. Exp. Biol. and Med.*, **39**, 591 (1938).
2. Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, **39**, 275 (1924). Todd, E. W., *Brit. J. Exp. Path.*, **11**, 368 (1930).

SUGAR ALCOHOLS

XX. THE FATE OF *d*-SORBITOL, STYRACITOL, AND *l*-SORBOSE IN THE ANIMAL BODY

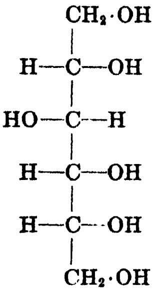
By C. JELLEFF CARR and SYLVAN E. FORMAN

(From the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore)

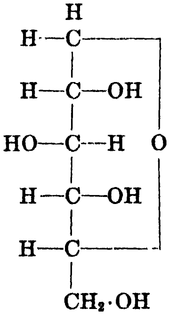
(Received for publication, February 8, 1939)

The sugar alcohols that have been previously studied generally serve as precursors of glycogen in rats and many are attacked by members of the colon *aerogenes* group of microorganisms with the production of acid and gas (1, 2). The anhydrides of these sugar alcohols are generally non-metabolizable by either microorganisms or the mammalian body. Recently we have observed that the anhydride, styracitol, was utilized by various members of the *Bacillus coli* group of organisms (3). To continue this study, the epimer of polygalitol, namely styracitol (1,5-anhydrosorbitol), was prepared in large quantities and experiments were conducted comparing the fate of this anhydride to sorbitol and the ketose, *l*-sorbose.

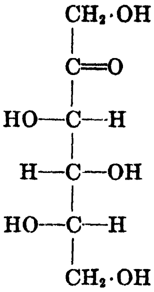
Approximately 100 gm. of styracitol were synthesized by the hydrogenation of tetraacetyloxyglucal and subsequent deacetylation of the reduction product according to the method of Zervas (4). The compound melted at 157° uncorrected; $[\alpha]_D^{24} = -48.2°$ (H_2O, $c = 1.5$); $[\alpha]_D^{20} = -53.4°$ (3.1 per cent H_3BO_3 in H_2O, $c = 3.3$). The *d*-sorbitol generously supplied by the Atlas Powder Company, Wilmington, was crystalline, non-reducing, with a melting point of 97°. The *l*-sorbose used in these studies was obtained through the kindness of Dr. P. A. Wells of the United States Department of Agriculture, m.p. 165°, $[\alpha]_D^{25} = -43.7°$ (H_2O, $c = 10$). The accompanying formulas show the relationship between the three compounds. Recent work by Freudenberg and Rogers (5) indicates that the structure of polygalitol is that which was assigned formerly to styracitol, namely 1,5-anhydrosorbitol, and that styracitol may be 1,5-anhydromannitol.



d-Sorbitol



1,5-Anhydrosorbitol



l-Sorbose

Glycogen Storage in Livers of White Rats—Male rats weighing between 100 and 150 gm. were fasted 48 hours and placed in individual cages in a constant temperature room at 25°. The control rats were fed a basal diet of cacao butter and the experimental animals mixtures of cacao butter (67 per cent) and the experimental compound (33 per cent). The feeding period varied from 50 to 120 hours as indicated in Table I. The animals were anesthetized with sodium amytal, the livers removed, and the glycogen determined by the Good *et al.* (6) modification of Pflüger's method. The dextrose was determined by the Shaffer and Hartmann (7) method. The details of the foregoing procedure have been reported previously (1). The results are shown in Table I.

The results indicate that all three compounds are utilized by the fasting rat to form additional glycogen in the liver. In addition it was observed that long feeding periods with these compounds increased the total amount of glycogen in the livers of the rats and the animals did not lose weight as did the control animals. The excreted urine and the urine removed from the bladder at the time of killing did not yield detectable quantities of styracitol upon analysis. In previous experiments with the unmetabolized anhydrides invariably these compounds could be detected unchanged in the excreted urine.

Influence of Styracitol on Respiratory Quotient—Using the open circuit method of determining respiratory metabolism as reported previously, we determined the influence of styracitol upon the fasting respiratory quotient of the white rat. As suggested by Chambers (8) the animals used in these studies were fasted 48 hours and lost approximately 12 per cent of their original body weight before the determination of the fasting R.Q. was made. The results as given in Table II indicate that the compound is not directly metabolized in the animal body as is dextrose which under the same experimental conditions gave an increase in the respiratory quotient from 0.716 to 0.800.

TABLE I

Glycogen Storage in Livers of Rats after Feeding d-Sorbitol, Styracitol, and l-Sorbose

No. of animals	Average food consumed		Feeding period	Liver glycogen		
				Maximal	Minimal	Mean
		gm.	*hrs.*	*per cent*	*per cent*	*per cent*
5	Cacao butter	8.5	72	0.17	0.03	0.13
10	Styracitol	1.6	50	0.80	0.20	0.40
4	Cacao butter	6.9	72	0.23	0.03	0.16
5	Styracitol	3.0	72	1.65	0.14	1.00
5	Cacao butter	7.8	69	0.25	0.04	0.11
6	Styracitol	2.5	69	0.77	0.15	0.35
2	"	3.5	93	2.36	1.77	2.06
5	Cacao butter	6.0	93	0.65	0.23	0.37
7	*l*-Sorbose	2.7	120	3.16	0.29	1.74
8	"	4.6	93	5.60	1.80	3.15
8	*d*-Sorbitol	3.8	89	2.36	0.21	1.16
5	"	4.2	93	1.96	0.55	1.37
5	Cacao butter	9.7	93	0.32	0.19	0.25

Styracitol and Blood Sugar Level—Freudenberg and Felton, during a study of the capacity of various carbohydrate substances to elevate the blood sugar level of rabbits, reported that styracitol was physiologically inactive in two experiments (9). In the present study the capacity of styracitol to elevate the blood sugar level has been reinvestigated when this compound is administered by stomach tube. Since the influence of both sorbitol and sorbose

TABLE II

Respiratory Quotients during Fasting and after Styracitol

Rat No.	Weight	Styracitol, 40 per cent solution, by stomach tube	O_2 per 100 gm. per hr.	R.Q.	Length of determination
	gm.	*gm.*	*gm.*		*hrs.*
43	121	Fasting	0.147	0.723	2.5
		1.4	0.181	0.728	2.5
43	134	Fasting	0.150	0.719	3.5
		1.4	0.151	0.741	3.4
54	140	Fasting	0.182	0.735	3.5
		1.4	0.178	0.751	2.5
53	117	Fasting	0.189	0.732	2.7
		0.5	0.189	0.710	2.5
52	112	Fasting	0.155	0.743	2.9
		0.4	0.185	0.704	2.5
50	127	Fasting	0.166	0.719	2.5
		0.4	0.179	0.700	2.5
59	123	Fasting	0.184	0.725	2.7
		0.4	0.193	0.716	2.5
55	124	Fasting	0.193	0.721	3.2
		1.4	0.191	0.705	2.5
108	120	Fasting	0.190	0.732	2.6
		0.4	0.192	0.725	3.5
152	132	Fasting	0.175	0.739	2.5
		1.4	0.188	0.726	5.0
Mean.............		Fasting	0.173	0.728	
		Styracitol	0.182	0.721	

TABLE III

Influence of Styracitol on Blood Sugar Level of Rabbits

Rabbit No.	Weight	Styracitol	Blood sugar, mg. per 100 cc. blood				Samples
			Fasting	Maximal	Minimal	Mean	
	kg.	*gm. per kg.*					
1	0.67	2.0	108	109	86	99	Every ½ hr. for 4 hrs.
2	0.80	1.0	110	122	106	110	" ½ " " 4 "
3	0.85	4.0	99	112	95	104	" ½ " " 4 "
4	0.95	2.0	98	123	94	111	" ½ " " 4 "
5	1.36	1.0	100	112	86	98	" 10 min. " 1 hr.

upon the blood sugar level after ingestion has been reported, these compounds were not used. The results are shown in Table III.

Acute Toxicity—The value of the minimum lethal dose of mannitol *per os* previously reported was found to be greater than 1.3 gm. per 100 gm. of body weight for rats. Owing to the greater solubility of sorbitol much larger amounts could be administered. Doses of 1.3 gm. of sorbitol per 100 gm. of animal produced only a mild depression and diarrhea in five animals. Quantities of 2.6 gm. per 100 gm. of body weight produced depression and death within 1 to 12 hours. Styracitol (two experiments) failed to produce any detectable effects in concentrations of 1.3 gm. per 100 gm. of rat. *l*-Sorbose in concentrations of 1.3 gm. per 100 gm. of rat (ten experiments) produced no toxic symptoms. It should be pointed out that these concentrations represent relatively massive amounts of the compound.

DISCUSSION

The data assembled indicate that the three chemically related compounds, *d*-sorbitol, styracitol, and *l*-sorbose give rise to additional glycogen storage in the livers of white rats. The effect was anticipated in the case of the ketose and the sugar alcohol, sorbitol, but the anhydride, styracitol, appears to be an exception to the general rule that anhydride formation among the sugar alcohols destroys glycogen-forming capacity. The sweet taste of this compound and its utilization by bacteria suggest utilization by the animal body. That styracitol is not directly metabolized is demonstrated by its inability to elevate the R.Q. and raise the blood sugar level of rabbits and indicates glycogen formation with subsequent utilization.

SUMMARY

1. *d*-Sorbitol, styracitol, and *l*-sorbose when fed in a basal fat diet for a period of days are stored as glycogen in the liver of the rat.

2. Styracitol does not raise the respiratory quotient or increase the O_2 consumption of the fasting rat when administered by stomach tube.

3. Styracitol does not elevate the fasting blood sugar level of the rabbit.

4. The results indicate that styracitol requires glycogen formation for utilization by the animal body.

5. These compounds are not toxic when administered by mouth to the white rat except in massive quantities.

BIBLIOGRAPHY

1. Carr, C. J., and Krantz, J. C., Jr., *J. Biol. Chem.*, **124**, 221 (1938).
2. Dozois, K. P., Carr, C. J., Krantz, J. C., Jr., Hachtel, F., and Beck, F. F., *J. Bact.*, **32**, 499 (1936).
3. Dozois, K. P., Carr, C. J., and Krantz, J. C., Jr., *J. Bact.*, **36**, 599 (1938).
4. Zervas, L., *Ber. chem. Ges.*, **63**, 1689 (1930).
5. Freudenberg, W., and Rogers, E. F., *J. Am. Chem. Soc.*, **59**, 1602 (1937).
6. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
7. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, **45**, 349 (1920–21).
8. Chambers, W. H., *Physiol. Rev.*, **18**, 248 (1938).
9. Freudenberg, W., and Felton, G. E., *J. Biol. Chem.*, **99**, 657 (1932–33).

DELPHININE

II. ON OXODELPHININE

By WALTER A. JACOBS and LYMAN C. CRAIG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, March 2, 1939)

Analytical results obtained with delphinine, the crystalline alkaloid from *Delphinium staphisagria*, L., have been shown by us (1) to correspond best with a formula $C_{33}H_{45}O_9N$ for this alkaloid. Its assumed close relationship to the aconite group of alkaloids has now been clearly demonstrated. In addition to four methoxyl groups it contains a benzoyl, an acetyl, and a free acylatable hydroxyl group and behaves as if it contains an N-alkyl (methyl) group. More recent studies have confirmed this analogy to the aconite alkaloids, since it loses acetic acid on pyrolysis to form a *pyrodelphinine*. It is probable that the hydroxyl and acetyl groups occur in a 1–4 or 1–5 relationship and that acetic acid is lost with the formation of an oxidic bridge. This has been indicated by the hydrogenation experiments in which only the benzoyl group appears to be involved.

The study of the oxidation of delphinine with permanganate has been carried further since the earlier report. In addition to the formation of the substance first described by Keller (2) and called by him *X-214°*, we have isolated a second apparently isomeric substance formed in smaller amount. By a modification of the earlier procedure a total yield of roughly 90 per cent has been obtained of the two isomers. For the reasons given below, the preponderating substance has been called *α-oxodelphinine* ($[\alpha]_D^{20} = -62°$ in acetic acid) and its companion *β-oxodelphinine* ($[\alpha]_D^{20} = +31°$). Many analyses of these substances, including our earlier published figures, are consistent with a formulation $C_{33}H_{43}O_{10}N$. This is a conclusion which has an important bearing on the interpretation of the correct formulation of oxonitine and its isomer (?), as discussed in the succeeding paper (3). Just as in the case of oxonitine and its companion, the isomeric oxodelphinines have lost the essential basic character of the parent alkaloid. Both the benzoyl and acetyl groups are retained, and on catalytic hydrogenation of the α isomer a *hexahydro-α-oxodelphinine* was formed. Although four methoxyl groups can still be readily determined, only a trace of an N-methyl could be detected. The above formulation $C_{33}H_{43}O_{10}N$, if correct, points to the oxidation of a CH_2 group adjoining the N atom to CO with the formation of a cyclic lactam. Either the so called N—CH_3 group is so modified by such a transformation that it eludes determination as an N—CH_3 group (4) or the so called N—CH_3 group of delphinine does not exist as such but as a larger grouping. The formation of methylamine from delphinine or methyl iodide during the N-alkyl determination would be difficult to explain except as the result of the cleavage of some larger grouping, such as a complex pyrrolidone, piperidone, or related derivatives. This is an inconsistency which can be cleared up only by further investigation.

The above formulation of α-oxodelphinine is supported by the analytical figures obtained with *pyro-α-oxodelphinine* formed from it on pyrolysis and with those given by *hexahydropyro-α-oxodelphinine* resulting from the latter on hydrogenation.. When α-oxodelphinine was heated with methyl alcoholic HCl, a crystalline neutral substance resulted. The analytical figures are in approximation to those required by a formula $C_{32}H_{43}O_9N$. The methoxyl determination indicates introduction of an extra methoxyl group (a total of five OCH_3 groups) and titration after saponification the presence of only one acyl group. It appears, therefore, that the acetyl group has been replaced by methyl. Simultaneously an appreciable amount of a basic fraction was isolated from the mixture which, however, has not been obtained in crystalline form. Since CO_2 was evolved in the reaction, the formation of this base possibly parallels that of the base obtained in a similar manner from oxonitine, as described in the succeeding paper (3).

EXPERIMENTAL

Pyrodelphinine—0.57 gm. of delphinine was heated for 10 minutes in an atmosphere of H_2 at 200–215°. The melted alkaloid evolved acetic acid. The resulting resin crystallized readily under ether and was collected in excellent yield. Recrystallized from 95 per cent alcohol, it formed pointed rods which slowly melted at 208–212° after preliminary softening. 0.28 gm. was so obtained. For analysis it was dried at 110° and 20 mm.

$C_{31}H_{41}O_7N$. Calculated, C 68.97, H 7.66; found, C 69.11, H 7.57

Pyrodelphinine readily absorbed H_2 in acetic acid solution with platinum oxide catalyst. After deducting for catalyst, the apparent absorption approximated 4 moles of H_2. The resulting substance could not be obtained in crystalline form.

α- and β-Oxodelphinine—Since our preliminary report on this substance, the following improved method for its preparation has been employed.

5 gm. of delphinine were dissolved in 500 cc. of dry acetone containing 5 cc. of acetic acid. 3 gm. of powdered potassium permanganate were then added and the mixture was left at about 25–30° overnight. The reagent was used up. The mixture was diluted with an equal volume of water, then treated with 40 cc. of 10 per cent H_2SO_4, and the MnO_2 was reduced with sulfur dioxide. The colorless crystals after collection with water gave a first fraction which weighed 2.85 gm. and melted at 218–221°. This fraction, which consisted essentially of α-oxodelphinine, was most readily recrystallized by solution in chloroform, addition of alcohol, and concentration to remove the chloroform. It formed flat needles which melted at 218–221°, depending on the rate of heating.

$$[\alpha]_D^{20} = -62° \text{ (}c = 1.05 \text{ in acetic acid)}$$
$$[\alpha]_D^{20} = -55° \text{ (}c = 0.275 \text{ in 95 per cent alcohol)}$$

Keller reported $[\alpha]_D^{20} = -52.2°$ ($c = 0.42$ in alcohol).

Numerous analyses of this substance have given consistent results. Our more recent ones are reported.

$C_{33}H_{43}O_{10}N$.	Calculated.		C 64.56,	H 7.07,	OCH_3 20.22
$C_{32}H_{41}O_{10}N$.	"		" 64.07,	" 6.89,	" 20.69
	Found.	(a)	" 64.72,	" 6.91,	" 19.88
	"		" 64.27,	" 7.12	
	"	(b)	" 64.44,	" 7.09	
	"	(c)	" 64.65,	" 7.13	

The mother liquor of the above first fraction which consisted of dilute acetone was further diluted and extracted with ether. The aqueous phase on being rendered alkaline and extracted further with ether yielded a very small fraction of delphinine which had not been oxidized. The above ether extract of oxidation products was repeatedly washed with water. On concentration it gave readily a second fraction of needles (0.62 gm.) which melted at 218–221°. The mother liquor of this fraction on further concentration gradually deposited a mixture of needles and stout four-sided prisms. A suspicion of the presence of the latter had already been obtained during the manipulation of the first main fraction. The yield of this third fraction was 0.48 gm. and consisted essentially of β-oxodelphinine. This melted somewhat higher at 222–224° after preliminary softening. The mother liquor of this fraction on still further concentration and dilution gave a viscous mass which gradually crystallized to a mass of small, stout rhombs. After collection with a little alcohol 0.35 gm. of this fraction was obtained, which melted at 228-229° with decomposition and appeared to be practically pure β-oxodelphinine. It was recrystallized as in the case of the first substance by boiling down the chloroform solution with alcohol. The melting point remained at 228–229°.

$[\alpha]_D^{20} = +31°$ ($c = 1.01$ in acetic acid)
$C_{33}H_{43}O_{10}N$. Calculated. C 64.56, H 7.07
Found. (*a*) " 64.47, " 6.83
" (*b*) " 64.53, " 6.85

Finally, from the last mother liquors a fifth and final fraction of similar crystals was obtained which amounted to 0.12 gm. The total yield of crystalline neutral material thus recovered from 5 gm. of delphinine was roughly 4.4 gm.

Hexahydro-α-Oxodelphinine—0.1 gm. of α-oxodelphinine was hydrogenated in acetic acid solution with 50 mg. of platinum oxide catalyst under 3 atmospheres pressure. The absorption of H_2 beyond the catalyst, 17 cc., was somewhat (about 1 mole) in excess of the 3 moles required, *viz.* 12 cc. The solution on concentration gave a resin which readily crystallized under ether. The collected material was recrystallized from ether and formed four-sided platelets which melted at 195°.

$C_{33}H_{49}O_{10}N$. Calculated. C 63.93, H 7.97
$C_{32}H_{47}O_{10}N$. " " 63.43, " 7.83
Found. " 63.74, " 7.75
" " 63.77, " 7.61

Pyro-α-Oxodelphinine—0.1 gm. of α-oxodelphinine was heated in an atmosphere of H_2 at 220° for 30 minutes. The resulting resin crystallized under ether. It was recrystallized from acetone and formed six-sided platelets which did not have a sharp melting point. The substance began to sinter above 150° and with gradual coloration slowly melted at 200–220°. It contained solvent. From methyl alcohol it formed flat needles which sintered to a resin above 238° and melted at 248–250°.

For analysis it was dried at 120° and 20 mm.

$C_{31}H_{39}O_8N$.	Calculated.	C 67.23,	H 7.10
	Found. (*a*)	" 67.20,	" 7.05
	" (*b*)	" 67.03,	" 7.05

When pyro-α-oxodelphinine was heated in a sealed tube with 30 parts of 3 per cent methyl alcoholic hydrochloric acid, it formed a clear solution which on concentration yielded a sparingly soluble substance. The latter separated from methyl alcohol as minute wedge-shaped crystals which melted slowly at 280–284° after preliminary softening above 270°. It was apparently an isomer of the above substance.

$C_{31}H_{39}O_8N$.	Calculated.	C 67.23,	H 7.10,	OCH_3 22.40
	Found.	" 66.72,	" 7.03,	" 21.84

Hexahydropyro-α-Oxodelphinine—Pyro-α-oxodelphinine was hydrogenated in acetic acid solution with platinum oxide catalyst. The apparent absorption of H_2 above the catalyst was excessive and between 4 and 5 moles. Recrystallized from a mixture of ether and petroleum ether the hydrogenation product formed needles which melted at 183–185° after preliminary sintering.

$C_{31}H_{45}O_8N$.	Calculated.	C 66.50,	H 8.11
	Found.	" 66.48,	" 8.22
	"	" 66.40,	" 8.27

α-Oxodelphinine and Methyl Alcoholic Hydrochloric Acid—0.5 gm. of α-oxodelphinine was heated for 18 hours in a sealed tube at 100° with 10 cc. of 3 per cent HCl in absolute methyl alcohol. The resulting clear solution was chilled in dry ice before the tube was opened and then, after opening, the evolved gases were allowed to pass through barium hydroxide solution. Definite evidence of CO_2 cleavage was obtained. The clear reaction mixture was concentrated to dryness *in vacuo* and the resulting residue crystallized readily from dilute alcohol. The first and second fractions amounted to about 0.2 gm. Recrystallized from a small volume of methyl alcohol, it separated as needles or long thin leaflets which on rapid heating melted at 220–222°. If the substance was first powdered, it sintered to a vitreous mass at this point and then melted at 235–236°. It appears that this behavior must be due to dimorphism, since the substance contained no appreciable amount of solvent of crystallization.

$C_{32}H_{43}O_9N$.	Calculated.	C 65.60,	H 7.40,	OCH_3 26.47
	Found.	" 65.32,	" 7.38,	" 24.62
	"	" 65.55,	" 7.22	
	"	" 65.25,	" 7.14	

14.64 mg. of substance were refluxed in a mixture of 3 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours and titrated back against phenolphthalein. Found, 0.27 cc.; calculated for 1 equivalent, 0.25 cc.

Under similar conditions 11.25 mg. of substance consumed 0.211 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 0.192 cc.

When the above substance was melted in an H_2 atmosphere in a bath at 260° and allowed to cool, it crystallized. After recrystallization from dilute methyl alcohol it formed needles and leaflets which from melting point and mixed melting point proved to be unchanged starting material.

Found. C 65.73, H 7.17, OCH_3 25.65

The dilute alcoholic mother liquor of the above neutral methylated derivative was acidified with dilute HCl and extracted with ether. The latter yielded a very small additional amount of crystalline material which was not investigated further. When the remaining aqueous phase was rendered alkaline with excess dilute Na_2CO_3 and extracted with ether, an appreciable fraction was obtained on concentration which had basic properties. All attempts to crystallize this material were unsuccessful. On drying down, it formed a colorless, brittle, triboelectric, vitreous mass. Although the homogeneity of the substance is in doubt, it was analyzed after drying at 100° and 20 mm.

Found. C 66.29, H 7.68, OCH_3 23.95, $N(CH_3)$ 0.81

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **127**, 361 (1939).
2. Keller, O., *Arch. Pharm.*, **263**, 274 (1925).
3. Jacobs, W. A., Elderfield, R. C., and Craig, L. C., *J. Biol. Chem.*, **128**, 439 (1939).
4. Späth, E., and Galinovsky, F., *Ber. chem. Ges.*, **64**, 2201 (1931). Kohn, M., and Ostersetzer, A., *Monatsh. Chem.*, **32**, 905 (1911). Herzig, J., and Lieb, H., *Monatsh. Chem.*, **39**, 285 (1918). Späth, E., *Monatsh. Chem.*, **40**, 29 (1919).

THE ACONITE ALKALOIDS*

II. THE FORMULA OF OXONITINE

BY WALTER A. JACOBS, ROBERT C. ELDERFIELD, AND LYMAN C. CRAIG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, March 2, 1939)

Since the discovery by Carr (2) in 1912 of oxonitine, the neutral, sparingly soluble product of the oxidation of aconitine with permanganate and for which he proposed a formula $C_{23}H_{29}O_9N$, difference of opinion has prevailed as to the correct formulation of this substance. This interpretation assumed a new aspect when Späth and Galinovsky (3) in 1930 suggested the formula $C_{32}H_{43}O_{12}N$ on the basis not only of the C and H determinations but also on those of N, methoxyl, and benzoic acid. This formulation was supported by the more recent analyses of Lawson (4). However, a further slight modification of the formula to $C_{32}H_{41}O_{12}N$ was made by Majima and Tamura (5) on the basis of evidence reported by them, which appeared to be conclusive. Both aconitine, $C_{34}H_{47}O_{11}N$, and mesaconitine (6), $C_{33}H_{45}O_{11}N$, had been found to yield on oxidation the same oxonitine. Aconitine, on the one hand, has been shown to behave as if it contained an N-ethyl group (1, 5) by the formation of ethyl iodide in the N-alkyl determination and of ethylamine on fusion with KOH. On the other hand, mesaconitine (5) appears to contain an N-methyl group, since after saponification to mesaconine and subsequent demethylation (of OCH_3 groups) methylamine was the principal volatile base obtained on alkali treatment of the resulting demethylated mesaconine. Oxonitine, when similarly transformed to a demethylated oxonine, gave mainly ammonia and since it no longer shows an N-alkyl group in the alkyl determination Majima and Tamura conclude that the transformation of aconitine and mesaconitine to oxonitine involves oxidative removal of the N-ethyl and N-methyl groups in these alkaloids with simultaneous oxidation of an adjoining CH_2 to CO group, with the formation of a lactam. The long known formation of acetaldehyde during the oxidation of aconitine to oxonitine and the detection by Majima and Tamura of formaldehyde among the oxidation products of mesaconitine seem to fit in with this interpretation. A precedent for the oxidative removal of an N-alkyl group is found in the conversion of tropine to tropigenine.

---

* Earlier work on the aconite alkaloids was reported in 1936 (1).

Thus a strong case appears to have been made for the nature of the transformation of these alkaloids into oxonitine. However, our recent experience may reopen this question. The analytical values which we have obtained with oxonitine prepared directly from Merck's crystalline aconitine as well as with aconitine purified over the hydrobromide according to the method of Majima and Suginome (7) have been consistently somewhat higher than those required by the formula $C_{32}H_{41}O_{12}N$ and in closer agreement with that of $C_{33}H_{43}O_{12}N$. This was supported by the analytical results obtained with the "isomer (?)"[1] previously described by us (1) and substantiated by our more recent work. At first we were reluctant to attribute too much significance to such analytical results until the experience with delphinine, as reported in the previous paper, had been encountered. Here from the analytical data the transformation of delphinine to oxodelphinine appears to proceed without loss of carbon atoms. Since this appeared to fit in with the oxonitine analyses, it was of importance to obtain other evidence. Accordingly, other derivatives of oxonitine were studied. On hydrogenation a *hexahydrooxonitine* was prepared. The analyses of this material supported a formula $C_{33}H_{49}O_{12}N$.

[1] Recent analyses of this more soluble oxidation product as well as those of its *hexahydro derivative* are in agreement with a formula $C_{34}H_{45}O_{12}N$ for the former. It must be formed from aconitine without loss of carbon atoms and is therefore an *oxoaconitine*. It is produced in larger amount than oxonitine. This at once brings up the question as to whether both oxonitine and oxoaconitine are simultaneously formed from aconitine itself by different reactions or whether the aconitine reported by different workers to yield oxonitine has not been homogeneous but contaminated by a C_{33} alkaloid which has been the source of oxonitine. This question is now under careful investigation.

Pyrooxonitine was then prepared according to Majima and Suginome (8). Contrary to the recent assumptions of Tamura (9), many analytical results which we have obtained with pyrooxonitine are in close agreement with a formula $C_{31}H_{39}O_{10}N$ rather than $C_{30}H_{37}O_{10}N$. Finally, on hydrogenation a crystalline *hexahydropyrooxonitine* was prepared, analysis of which supported the formula $C_{31}H_{45}O_{10}N$.

Thus from our experience the question of the exact relationship of oxonitine to aconitine has been reopened and can be finally settled only when the exact nature of the steps involved in the transformation of mesaconitine and aconitine to oxonitine has been determined.

In accordance with this, attempts at the degradation of oxonitine have been in progress. At this point we wish to report the results of a preliminary study of the cleavage of oxonitine with methyl alcoholic HCl. Roughly 40 to 50 per cent of a crystalline base has been obtained. Analyses indicate the presence of five methoxyl groups and at least one N-methyl group. Since CO_2 was formed during the production of the substance, the possibility appears that lactam cleavage occurs with loss of CO_2 and either with or without methylation of a resulting secondary amino group. Since in the case of the reaction of oxodelphinine with methyl alcoholic HCl the acetyl group is replaced by methyl, such a reaction may also occur here. The resulting base should then have a formula $C_{31}H_{45}O_{10}N$ or $C_{32}H_{47}O_{10}N$. However, this interpretation can be merely provisional and must await the results of further study. Attempts at further degradation (exhaustive methylation) of the substance are now in progress.

EXPERIMENTAL

Oxonitine—For the preparation of this substance we have used Merck's crystalline aconitine directly as such, and aconitine obtained from it by purification over the hydrobromide according to Majima and Suginome (7). In the latter case the recrystallized hydrobromide was reconverted to the base which was then recrystallized from methyl alcohol. This material then melted at 202–205°, depending on the rate of heating.

$C_{34}H_{47}O_{11}N$. Calculated, C 63.22, H 7.34; found, C 63.40, H 7.28

The purified alkaloid was oxidized with permanganate in acetone and acetic acid solution essentially according to the procedure of Barger and Field (10). After completion of the oxidation, the collected mixture of MnO_2 and oxonitine which had crystallized was treated in aqueous suspension with SO_2 to remove the MnO_2. From 5 gm. of purified aconitine, 0.83 gm. of oxonitine was directly obtained. After recrystallization by addition of acetone to the solution in hot acetic acid, it separated as the usual heavy crystalline powder and melted at 279–282° after preliminary softening.

$[\alpha]_D^{20} = -45°$ ($c = 0.956$ in chloroform)
$C_{33}H_{43}O_{12}N$. Calculated. C 61.36, H 6.72
$C_{32}H_{41}O_{12}N$. " " 60.82, " 6.55
Found. " 61.67, " 6.61

Other preparations of equivalent character gave the following results.

Found. C 61.39, H 6.71
" " 61.53, " 6.63

When Merck's crystalline aconitine was directly employed without purification, similar results were obtained.

Found. C 61.45, H 6.65
" " 61.24, " 6.54
" " 61.09, " 6.70
" " 61.00, " 6.48

From 20 gm. of Merck's alkaloid, the yield of crude oxonitine was 5.5 gm. and 4.1 gm. of the following substance.

The acetone filtrate from the mixture of MnO_2 and oxonitine obtained above from the oxidation of recrystallized aconitine was concentrated to about 20 cc. On dilution with water an appreciable precipitate formed, which was collected with water. It proved to be a mixture. The filtrate from this after neutralization with sodium carbonate solution was extracted with chloroform. The washed chloroform extract was dried and concentrated to dryness. The residue when dissolved in a small volume of methyl alcohol slowly deposited prisms and rods. The yield of collected material was 1.3 gm. After recrystallization from methyl alcohol it melted at 261°. The substance, like oxonitine, is not dissolved by dilute acid or alkali.

$[\alpha]_D^{26} = -98°$ ($c = 0.956$ in chloroform)

$C_{33}H_{43}O_{12}N$.	Calculated.	C 61.36,	H 6.72,	N 2.17,	OCH_3	19.22
	Found. (*a*)	" 61.40,	" 6.69,	" 2.57,	"	19.18
	" (*b*)	" 61.45,	" 6.75,	" 2.42,	"	19.07

From a run of 20 gm. of Merck's crystalline aconitine the yield of this substance was 4.1 gm. After recrystallization from methyl alcohol it melted at 261°.

$[\alpha]_D^{25} = -99°$ ($c = 1.01$ in chloroform)
Found. C 61.69, H 6.51

Hexahydroaconitine[2]—0.5 gm. of purified aconitine was dissolved in 5 cc. of alcohol with a few drops of HCl and hydrogenated with 50 mg. of platinum oxide catalyst under 3 atmospheres pressure. The absorption of H_2 was prompt and appeared to be complete in about 30 minutes. The absorption corresponded to 3 moles. The hydrogenated alkaloid as such was not obtained in crystalline form but was isolated as the beautifully crystalline perchlorate. This was obtained by addition of sodium perchlorate solution to the aqueous solution of the very soluble hydrochloride. Recrystallized by dilution of its concentrated solution in alcohol, it formed square-ended prisms which melted at 209–210° after preliminary sintering.

$C_{34}H_{53}O_{11}N \cdot HClO_4$.	Calculated.	C 54.26,	H 7.24
	Found.	" 53.84,	" 7.35

On hydrolysis with water at 160–165° hexahydrobenzoic acid was cleaved from it. After acidification with HCl and extraction with ether, this acid was obtained as an oil with characteristic odor. The concentrated HCl solution after addition of acetone crystallized on seeding with aconine hydrochloride. The collected material melted at 174–176° and gave no depression with aconine hydrochloride.

Hexahydrooxonitine—0.15 gm. of oxonitine was suspended in acetic acid and shaken with 50 mg. of platinum oxide catalyst in hydrogen under 3 atmospheres pressure. Although absorption occurred promptly and all oxonitine dissolved within several hours, the operation was continued overnight. The filtrate from the catalyst left on concentration a crystalline residue. The substance formed prisms or minute rods from 95 per cent alcohol, which melted at 253° on rapid heating.

---

[2] Without isolation of the products, Freudenberg (11) demonstrated the absorption of 3 moles of H_2 by the benzoyl group in aconitine and oxonitine, and not in aconine.

$C_{33}H_{49}O_{12}N$.	Calculated.	C 60.80,	H 7.58
$C_{32}H_{47}O_{12}N$.	"	" 60.25,	" 7.43
	Found.	" 60.74,	" 7.49
	"	" 60.94,	" 7.30

Pyrooxonitine—This was prepared essentially according to Majima and Suginome (8). 0.4 gm. of oxonitine was heated in an atmosphere of H_2 at 280–285°. As soon as the substance melted, the tube was withdrawn from the bath. After cooling, the melt was dissolved in a few cc. of methyl alcohol and allowed to stand at 25° overnight. A small amount of unchanged oxonitine separated. The concentrated filtrate gave a resin which crystallized readily under chloroform. The collected material was recrystallized from chloroform and contained solvent. It was collected with chloroform and washed with a mixture of chloroform and petroleum ether.

For analysis it was dried at 120° and 20 mm.

$C_{31}H_{39}O_{10}N$.	Calculated.	C 63.55,	H 6.72
$C_{30}H_{37}O_{10}N$.	"	" 63.01,	" 6.53
	Found.	" 63.48,	" 6.77
	"	" 63.68,	" 6.90

In another experiment successive fractions were obtained from chloroform, which after recrystallization from chloroform gave the following figures.

Found.	(*a*)	C 63.30,	H 6.45
"	(*b*)	" 63.34,	" 6.85

Fraction (*a*) was then recrystallized from a mixture of alcohol and ether.

Found.	C 63.45,	H 6.77
"	" 63.53,	" 6.78

Majima and Suginome have reported a melting point of 231° for pyrooxonitine. In no case have we been able to duplicate this. Our substance as it separated from chloroform melted slowly to a colorless resin at 160–170°. The material from alcohol-ether, which lost about 1 per cent in weight on drying, softened gradually to a resin at 170–180° which melted on further heating. Finally, when recrystallized from methyl alcohol the substance melted sharply at 180°. In the latter case a rotation was found of $[\alpha]_D^{25} = -127°$ ($c = 1.14$ in methyl alcohol). This agrees closely with the values reported by Majima and Suginome, which approximated $[\alpha]_D = -128°$ in methyl alcohol.

Hexahydropyrooxonitine—0.15 gm. of pyrooxonitine was hydrogenated in alcoholic solution under 3 atmospheres pressure with 50 mg. of platinum oxide catalyst. After 2½ hours absorption had practically stopped. The absorption due to the substance was slightly in excess of 3 moles. The concentrated filtrate crystallized readily under ether. The collected material was recrystallized from acetone-ether and formed platelets which melted slowly at 160–163° to a viscous mass.

For analysis it was dried at 110° and 20 mm.

$C_{31}H_{45}O_{10}N$.	Calculated.	C 62.91,	H 7.67
$C_{30}H_{43}O_{10}N$.	"	" 62.35,	" 7.51
	Found.	" 62.81,	" 7.67
	"	" 62.76,	" 7.53

Base, from Oxonitine—50 mg. of oxonitine were heated in a tube with 2 cc. of a 6 per cent solution of HCl in dry methyl alcohol at 100° for 18 hours. A clear solution resulted which was concentrated *in vacuo* to dryness. The resinous residue was dissolved in water and after being made alkaline with dilute Na_2CO_3 the mixture was extracted with chloroform. The dried extract on concentration gave a residue which crystallized as platelets from ethyl acetate. The yield was 25 mg. The substance proved to be a base and dissolved readily in dilute acid. It melted at 250° after preliminary sintering. It was easily soluble in alcohol, acetone, less readily in ethyl acetate, and but sparingly soluble in ether.

$C_{31}H_{45}O_{10}N$.	Calculated.	C 62.91,	H 7.67,	N 2.37
$C_{32}H_{47}O_{10}N$.	"	" 63.43,	" 7.83,	" 2.31
	Found.	" 62.49,	" 7.58	
	"	" 62.97,	" 7.61,	" 2.61
$C_{31}H_{45}O_{10}N$.	Calculated.	5(OCH_3) 26.22,	N(CH_3) 2.54	
$C_{32}H_{47}O_{10}N$.	"	" 25.62,	" 2.49	
	Found.	" 24.94,	" 3.19	

In another experiment in which 1 gm. of oxonitine was heated with 25 cc. of 4.7 per cent methyl alcoholic HCl, the tube was chilled to low temperature before opening. On opening, it was at once equipped with a tube leading into $Ba(OH)_2$ solution. As the tube warmed up, the presence of CO_2 in the evolved gases was readily noted. No attempt at a quantitative estimation was made. In this experiment the yield of base was 0.36 gm.

BIBLIOGRAPHY

1. Jacobs, W. A., and Elderfield, R. C., *J. Am. Chem. Soc.*, **58**, 1059 (1936).
2. Carr, F. H., *J. Chem. Soc.*, **101**, 2241 (1912).
3. Späth, E., and Galinovsky, F., *Ber. chem. Ges.*, **63**, 2994 (1930).
4. Lawson, A., *J. Chem. Soc.*, 80 (1936).
5. Majima, R., and Tamura, K., *Ann. Chem.*, **526**, 116 (1936).
6. Morio, S., *Ann. Chem.*, **476**, 181 (1929).
7. Majima, R., and Suginome, H., *Ber. chem. Ges.*, **57**, 1466 (1924).
8. Majima, R., and Suginome, H., *Ber. chem. Ges.*, **58**, 2049 (1925).
9. Tamura, K., *Ann. Chem.*, **533**, 183 (1937).
10. Barger, G., and Field, E., *J. Chem. Soc.*, **107**, 231 (1915).
11. Freudenberg, W., *Ber. chem. Ges.*, **69**, 1962 (1936).

INHIBITION OF RESPIRATION AND PHOTOSYNTHESIS IN CHLORELLA PYRENOIDOSA BY ORGANIC COMPOUNDS THAT INHIBIT COPPER CATALYSIS*

By LOWELL F. GREEN, JAMES F. McCARTHY, and C. G. KING

(*From the Department of Chemistry, University of Pittsburgh, Pittsburgh*)

(Received for publication, February 23, 1939)

The suggestion that compounds known to inhibit copper catalysis might exert an inhibiting effect upon photosynthesis and respiration in green plants arose primarily from the following considerations: (*a*) The aerobic oxidation of ascorbic acid is very sensitive to catalysis by copper-protein complexes in a great variety of plant press-juices (1). (*b*) Both copper (2) and ascorbic acid (3) are found in relatively high concentrations in green leaves and other plant tissues that have a capacity for photosynthesis. (*c*) Although the evidence is far from complete, it is not unreasonable to believe that copper and ascorbic acid may be concerned, directly or indirectly, with both respiration and photosynthesis in green plants.

Previous work in other laboratories has shown that photosynthesis is more sensitive than respiration to inhibition by cyanide (4–6) and the urethanes (6, 7).

The present investigation has shown that typical copper "poisons" inhibit both photosynthesis and respiration in *Chlorella* cells, the respiration being less sensitive than photosynthesis.

EXPERIMENTAL

The unicellular fresh water alga *Chlorella pyrenoidosa* was grown in pure culture and used for the experiments primarily because of the extensive investigations that have been made on photosyn-

---

* Contribution No. 372 from the Department of Chemistry, University of Pittsburgh.

The authors are indebted to the Buhl Foundation for a research grant that has made the present investigation possible.

thesis and respiration in these plants, and further, because of the convenience of their use in manometric vessels. The strain selected has been in use for a number of years by one of us (8, 9) as well as by others (5–7, 10–13), for photosynthesis experiments.

The method of culture was essentially the same as that used by the authors cited. Flasks containing sterile salt solutions were inoculated and placed in a water bath close to an ordinary incandescent lamp (60 watt). A slow continuous stream of 5 per cent carbon dioxide in air was passed through the flasks. The temperature of the water bath was maintained at about 25°. The cultures were used after they had grown for 2 or 3 weeks, at which time they contained about 4 c.mm. of cells (centrifuged) per ml. of fluid.

The manometric method of Warburg (6) was used to determine the rates of respiration and of photosynthesis during short periods. Vessels of the rectangular type were employed, with a fluid volume of 8 ml. For the experiments the *Chlorella* cells were centrifuged out of their culture fluid and suspended in 0.05 M KH_2PO_4, pH 4.6, which was not altered when the suspension was saturated with 5 per cent CO_2 in air for the photosynthesis experiments. This phosphate-CO_2 medium has been found very favorable for measurement and calculation of maximum photosynthesis rates (9).

For attaining the high light intensities required to promote a maximum rate of photosynthesis, incandescent lamps (60 watt) were immersed in the thermostat very close to the vessels, one lamp for each vessel. This was found to give adequate intensity, but made the control of temperature difficult at 25°. Because of this, most of the experiments, including all of those specifically cited here, were performed at 30°. Control experiments showed a constant rate of photosynthesis over a period of at least 2 hours. In the respiration experiments the vessels were covered with tin-foil.

The calculation of rates is based on the assumption that the respiratory and photosynthetic quotients ($-\,CO_2/O_2$) are unity. This is known to be true in the absence of the inhibitors (6, 14, 15). The inhibitors may have some effect on these quotients and in turn on the recorded quantitative rates, but the error would probably not alter the interpretation of our results. Assuming unity of these quotients, the amount of O_2 produced or consumed (c.mm.)

is equal to $K_1 \times h$, where h is the pressure change in mm. and K_1 is the vessel constant for a quotient of unity, equal to $k_{CO_2} \times$

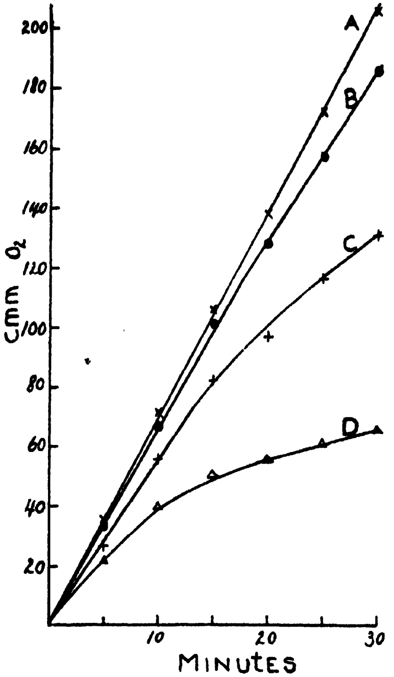


Fig. 1

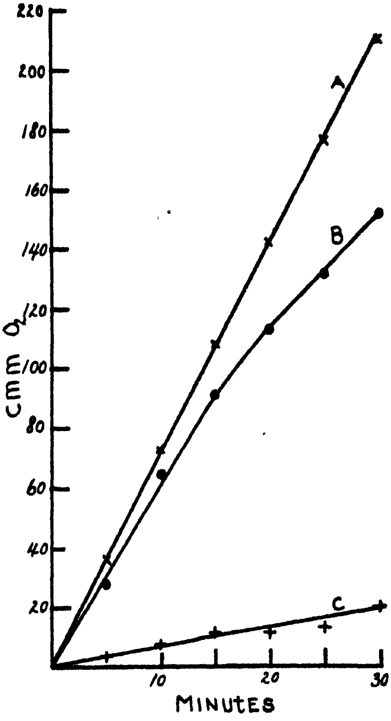


Fig. 2

Fig. 1. Inhibition of photosynthesis in *Chlorella* by thiourea. Temperature 30°, pH 4.6, 0.05 M KH_2PO_4, 20 c.mm. of cells, 8 ml. total fluid volume per vessel. Curve A, control, no inhibitor; Curve B, 0.035 mM of inhibitor; Curve C, 0.07 mM of inhibitor; Curve D, 0.14 mM of inhibitor.

Fig. 2. Inhibition of photosynthesis in *Chlorella* by 8-hydroxyquinoline. Temperature 30°, pH 4.6, 0.05 M KH_2PO_4, 20 c.mm. of cells, 8 ml. total fluid volume. Curve A, control, no inhibitor; Curve B, 0.01 mM of inhibitor; Curve C, 0.061 mM of inhibitor.

$k_{O_2}/k_{CO_2} - k_{O_2}$, k_{CO_2} and k_{O_2} being the vessel constants for a single gas, calculated in the usual manner (14, 15).

Effect of Inhibitors—Typical photosynthesis experiments are shown in Figs. 1 and 2. Each curve represents a separate vessel, the volume of cells in each vessel being 20 c.mm. (centrifuged). The amounts of thiourea and of 8-hydroxyquinoline shown are the total amounts in 8 ml. of fluid. The inhibitors were added to the cell suspensions about 50 minutes before the pressure readings were begun.

For the measurement of respiration a much larger volume of cells was required, as the respiratory rate of the *Chlorella* cells was about 2.5 per cent of the photosynthesis rate.

The amounts of inhibitors required to inhibit respiration were larger than required for inhibiting photosynthesis, partly because of the larger number of cells present, and partly because of a lower sensitivity of respiration to the inhibitors. Small amounts of the inhibitors were either without effect or produced an acceleration of respiration. Fig. 3 illustrates the effect of thiourea on respiration. The thiourea was added to 100 c.mm. of cells 35 minutes before the readings were begun. The amounts of inhibitors shown are the total amounts in 8 ml. of fluid.

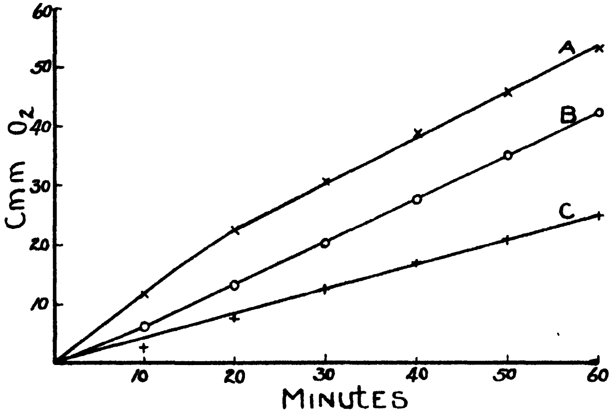


FIG. 3. Inhibition of respiration in *Chlorella* by thiourea. Temperature 30°, pH 4.6, 0.05 M KH_2PO_4, 100 c.mm. of cells. Curve A, no inhibitor; Curve B, 0.7 mM of inhibitor; Curve C, 1.5 mM of inhibitor.

Because of the low solubility of 8-hydroxyquinoline, it was not possible to obtain a solution concentrated enough to inhibit respiration. A saturated solution gave a rate identical with that of the control.

The other inhibitors tried, potassium ethyl xanthate, sodium diethyldithiocarbamate, allylthiourea, and salicylaldoxime, all gave marked inhibitions of photosynthesis when similar amounts of the inhibitors were present. The effects of xanthate and carbamate on respiration were obscured by the evolution of gas in the presence of large amounts of *Chlorella* cells. This was sufficient to overbalance the negative pressure change due to respiration, and was apparently due to a slow decomposition of these rather unstable organic substances.

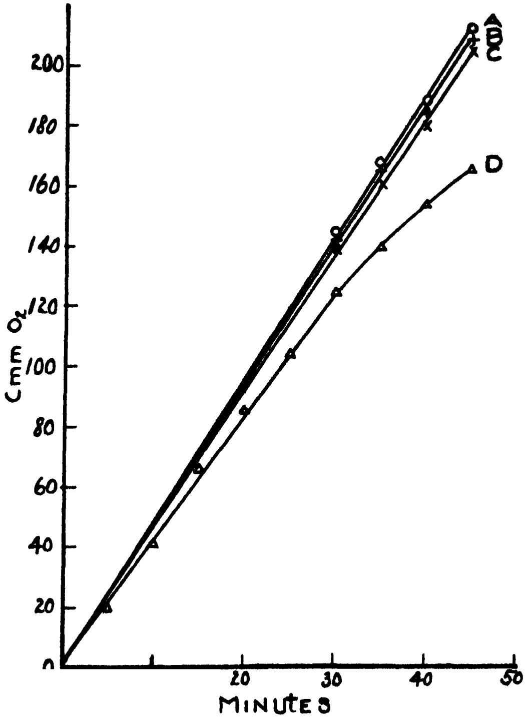


Fig. 4. Reversibility of thiourea inhibition of photosynthesis in *Chlorella*. Temperature 30°, pH 4.6, 0.05 M KH$_2$PO$_4$, 20 c.mm. of cells. Curve A, dialyzed cells, no inhibitor; Curve B, 0.07 mM of inhibitor added, then dialyzed; Curve C, undialyzed cells, no inhibitor added; Curve D, 0.07 mM of inhibitor added, without dialysis.

Reversibility of Inhibitions—Studies were made concerning the reversibility of thiourea and salicylaldoxime inhibition on photosynthesis and respiration. Aliquot portions of the necessary volume of culture suspended in 0.05 M KH_2PO_4 (pH 4.6) were used. One was poisoned, then dialyzed against water distilled in Pyrex vessels. Another dialyzed similarly but without the addition of an inhibitor, served as a control. A third portion was used as an undialyzed control, for comparison with the fourth portion to which the inhibitor was added. The results with thiourea as an inhibitor of photosynthesis are illustrated in Fig. 4. The inhibitor was left in contact with the cells for 30 to 40 minutes before dialysis was begun. A period of 4 hours of dialysis in cellulose (Visking) tubes, with frequent changes of water, was sufficient to remove the inhibitors to non-effective concentrations. The cell suspensions were centrifuged and brought to their original volumes with 0.05 M KH_2PO_4 before being placed in the vessels. The reversibility of salicylaldoxime inhibition is shown in Table I. As can be seen from Fig. 4 and Table I, dialysis (alone) of the *Chlorella* suspension caused slight increases in rates of respiration and photosynthesis.

TABLE I

Reversibility of Salicylaldoxime Inhibition of Chlorella Respiration and Photosynthesis

Temperature 30°, pH 4.5, 0.05 M KH_2PO_4. The results are given in c.mm. of O_2 per hour.

Inhibitor	Respiration		Photosynthesis	
	Inhibited	After dialysis	Inhibited	After dialysis
None	45 (initial)	63	58 (initial)	67
Salicylaldoxime*	8	61	25	65

* The amount of salicylaldoxime used in the respiration experiments was 0.043 mM with 100 c.mm. of cells; in photosynthesis, 0.014 mM with 20 c.mm. of cells. These quantities represent the total amounts in 8 ml., the total volume in the vessels.

A number of titrations (3, 16) of the ascorbic acid content of the centrifuged *Chlorella* cells gave an average value of 0.17 mg. per ml., without marked variation in the samples studied.

SUMMARY

1. A series of organic compounds characterized by their activity as copper "poisons," thiourea, 8-hydroxyquinoline, allylthiourea, sodium diethyldithiocarbamate, potassium ethyl xanthate, and salicylaldoxime all exhibited marked inhibition of photosynthesis in *Chlorella pyrenoidosa.*

2. The respiration of *Chlorella pyrenoidosa* was also inhibited by thiourea and salicylaldoxime, although higher concentrations were required, as found previously for cyanide inhibition. The solubility of 8-hydroxyquinoline was too low to permit a marked effect upon respiration. The effect of sodium diethyldithiocarbamate and potassium ethyl xanthate on respiration was obscured by the effect of their decomposition products.

3. The inhibition of both respiration and photosynthesis in *Chlorella pyrenoidosa* by thiourea and salicylaldoxime was reversible. The initial rates were fully restored after 4 hours dialysis and centrifuging.

4. The ascorbic acid content of centrifuged cells of *Chlorella pyrenoidosa* was 0.17 mg. per ml.

BIBLIOGRAPHY

1. Stotz, E., Harrer, C. J., and King, C. G., *J. Biol. Chem.*, **119**, 511 (1937).
2. Elvehjem, C. A., *Physiol. Rev.*, **15**, 471 (1935).
3. Bessey, O. A., and King, C. G., *J. Biol. Chem.*, **103**, 687 (1933).
4. van der Paauw, F., *Planta*, **24**, 353 (1935).
5. Briggs, G. E., *Proc. Roy. Soc. London, Series B*, **105**, 1 (1929).
6. Warburg, O., *Biochem. Z.*, **100**, 230 (1919).
7. Warburg, O., *Biochem. Z.*, **103**, 188 (1920).
8. Emerson, R., and Green, L. F., *J. Gen. Physiol.*, **17**, 817 (1934).
9. Emerson, R., and Green, L. F., *Plant Physiol.*, **13**, 157 (1938).
10. Warburg, O., and Negelein, E., *Biochem. Z.*, **110**, 66 (1920).
11. Warburg, O., and Negelein, E., *Z. physik. Chem.*, **102**, 235 (1928).
12. French, C. S., *J. Gen. Physiol.*, **18**, 209 (1934).
13. Baly, E. C. C., *Nature*, **134**, 933 (1934).
14. Warburg, O., Über den Stoffwechsel der Tumoren, Berlin (1926).
15. Warburg, O., and Negelein, E., *Z. physik. Chem.*, **106**, 191 (1923).
16. Musulin, R. R., and King, C. G., *J. Biol. Chem.*, **116**, 409 (1936).

THE SUBSTRATE SPECIFICITY AND INHIBITION CHARACTERISTICS OF TWO COPPER-PROTEIN "OXIDASES"*

BY JAMES F. McCARTHY, LOWELL F. GREEN, AND C. G. KING

(From the Department of Chemistry, University of Pittsburgh, Pittsburgh)

(Received for publication, February 23, 1939)

Evidence has been presented in earlier publications that the rapid oxidation of ascorbic acid in plant press-juices, when exposed to air, is primarily due to the catalytic effect of copper combined with protein. The evidence was of three types: (*a*) sensitivity to a number of organic and inorganic copper inhibitors, (*b*) the presence of adequate quantities of copper in the press-juices and enzyme preparations, and (*c*) the similarity between the enzyme preparations and known copper-protein complexes (1, 2).

Other plant and animal oxidative catalysts, such as (*a*) those that lead to quinone formation, (*b*) the cytochrome system, and (*c*) hemochromogens, can obviously serve as catalysts for the oxidation of ascorbic acid, but the dominant active agent in many plants appears to be copper.

In view of the subsequent finding by Kubowitz that potato "polyphenol oxidase" was also a copper-protein type of catalyst (3), it was of interest to investigate the relative substrate specificities and inhibition characteristics of these two forms of copper-protein.

Previous evidence has indicated that neither type of catalyst is highly specific in relation to substrates. We have pointed out the undesirability of naming the catalyst that serves for the oxidation of ascorbic acid in terms of a specific substrate, at least until further evidence of its substrate specificity is available (1), and Kubowitz

---

* Contribution No. 373 from the Department of Chemistry, University of Pittsburgh.

The authors are indebted to the Buhl Foundation for a research grant that has made this investigation possible.

has urged a non-specific type of nomenclature in relation to the highly purified oxidase from potato (4). The preparation studied by Johnson and Zilva (5) was active on the following substrates: *l*-ascorbic acid (vitamin C), *d*-ascorbic acid, *d*- and *l*-glucoascorbic acid, *d*- and *l*-galactoascorbic acid, and *d*- and *l*-araboascorbic acid (the names being derived from the corresponding sugar to which 1 carbon atom had been added by a Kiliani synthesis). Kubowitz's enzyme (3, 4) was active on catechol, tyrosine, adrenalin, dihydroxycinnamic acid, dopa, pyrogallol, and protocatechuic acid. After an induction period, phenol and *p*-cresol also served as substrates.

Rosenberg, using a titrimetric measure of oxidation rate, reported (6) that both *d*-araboascorbic and *d*-ascorbic acids were oxidized at a slower rate than *l*-ascorbic acid. Zilva has found *l*-ascorbic and *d*-araboascorbic, or *d*-isoascorbic acid to be oxidized at the same rate.

EXPERIMENTAL

The water used for rinsing apparatus and making up solutions was redistilled three times from Pyrex stills, the first containing alkaline permanganate, and the second, phosphoric acid. Kahlbaum's phosphates were used in making up buffers. The catechol was resublimed and potassium ethyl xanthate was freshly prepared. All other substrates and inhibitors were recrystallized to sharp melting points.

The manometric methods of Warburg, with Erlenmeyer type, air-filled vessels, were used in all of the experiments. The enzymes and copper salt solutions were placed in the main vessel, with substrates in the side arm and 0.2 ml. of 10 per cent KOH in the alkali cup. The temperature was 37.2° and the total volume in each cup was 3.4 ml. The rate of reaction was expressed as c.mm. of O_2 consumed per hour, extrapolated when necessary from the linear rate obtained for the major part of the reaction.

The experimental copper-protein solutions contained sufficient copper to give an oxidation rate comparable to that of the enzymes. Studies were made with both slow and fast rates of oxidation.

Although the buffers contained sufficient copper to cause a slow oxidation of ascorbic and isoascorbic acids, this effect was negligible in the presence of protein.

Preparation of Enzymes—The cucumber juice, pH 5.9 ± 0.1, was pressed from the previously frozen pericarp immediately after thawing in a glass mortar. After centrifuging, enzyme concentrates were prepared by acetone precipitation and by lowering the pH of the juice. In the former method, as recorded in the general procedure of Tauber *et al.* (7) for preparing the enzyme from squash, the active precipitate separated when the acetone concentration was between 20 and 40 per cent. The separation was not sharp, however, and the product underwent extensive denaturation. Peculiarly, the suspensions were often nearly as active as the original solutions. Fractional precipitation by acids was, in general, more satisfactory than the acetone fractionation. Inactive proteins separated when the pH was lowered to 5.0, followed by precipitation of the active fraction between 5.0 and 4.5. Redispersion was nearly complete at pH 9.0, and could be followed by repeated precipitations at pH 4.5.

The potato oxidase was prepared according to the method described by Kubowitz (3).

The experimental copper-protein complex was prepared by adding dilute copper sulfate solution to a 5 per cent solution of egg albumin, followed by dilution.

Substrate Specificity—As indicated by the data in Fig. 1, in agreement with the finding by Johnson and Zilva (5), the cucumber oxidase was found to be equally active on *l*-ascorbic acid and *d*-isoascorbic acid. The latter (Curves A, C, and E) was oxidized slightly faster than ascorbic acid (Curves B, D, and F) when copper ion or synthetic copper-protein catalysts were used.

Under the same experimental conditions (as in Fig. 1), except for the added substrates, *p*-phenylenediamine, kojic acid, and catechol did not cause an appreciable oxygen uptake in the presence of the same three catalysts. Copper ion was tested at concentrations as high as 0.11 mM.

Very active potato oxidase preparations had no effect on kojic acid, *p*-phenylenediamine, ascorbic acid, or *d*-isoascorbic acid. The failure of ascorbic acid to serve as a substrate for the potato oxidase was also observed by Kubowitz in his latest paper (4).

The inhibitors were neutralized when necessary and diluted to the desired concentrations with 0.1 M Sørensen phosphate buffer (pH 5.9).

As shown in Table I, the effect of the inhibitors was similar on the cucumber enzyme (acting on the above substrates) and on the potato oxidase (catechol substrate). Salicylaldoxime inhibition was not complete on either cucumber or potato oxidase at high concentrations of the enzymes, but when the oxidation rates were decreased by dilution, fairly low concentrations of the "poison" gave complete inhibition. Although 8-hydroxyquinoline gave consistent results with dilute protein solutions, we again noted its inability to block copper-albumin or enzymic catalysis when the protein concentration was high. The concentrations of inhibitors reported in Table II are much higher than the concentrations required to produce complete inhibition in the presence of low protein concentrations. Ethyl urethane exerted practically no effect at comparable concentrations.

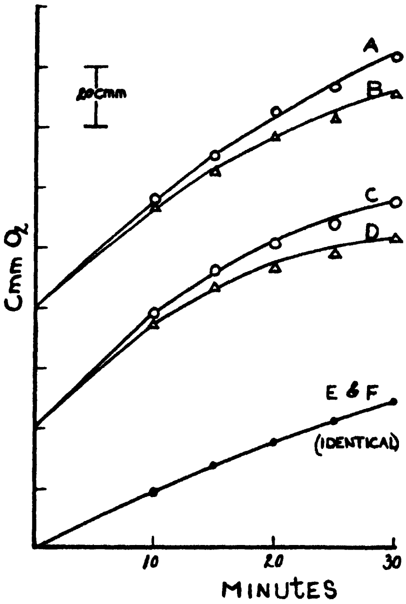


FIG. 1. The catalytic effect of copper ion, copper-protein, and cucumber oxidase on the aerobic oxidation of *d*-isoascorbic (Curves A, C, and E) and *l*-ascorbic (Curves B, D, and F) acids. Curves A and B, 6×10^{-6} mM of Cu with protein; Curves C and D, 6×10^{-6} mM of Cu alone; Curves E and F (identical), cucumber oxidase. *T* 37.2°, pH 5.9, 0.1 M PO_4 buffer, 5.6×10^{-3} mM of substrate.

Dialysis—Both the potato oxidase and the cucumber oxidase were dialyzed against triple distilled water at pH 5.8 to 5.9 for as long as 12 days without a marked loss of activity. At pH values of 3 or less, dialysis caused a distinct loss in activity in much less time. Kubowitz reported the removal of copper from potato oxidase under similar conditions. When inactivated by sodium cyanide, the cucumber enzyme remained inactive after dialysis, a peculiarity that will be further investigated. This was in contrast to the effect of dialysis upon potato oxidase, whose activity was restored by removal of the cyanide. Dialysis at pH 5.9 restored the activity of both enzymes following their inactivation by diethyldithiocarbamate and thiourea. The results of these investigations are shown in Table III. The pH was regulated to 5.9 ± 0.1 throughout the experiments.

TABLE I

Substrate Specificity and Effect of Copper Inhibitors

	Rate of O_2 consumption, c.mm. O_2 per hr. (pH 5.9, 37.2°)					
	Ascorbic acid, 0.01 mM				Catechol, 0.007 mM	
	3×10^{-6} mM Cu^{++}	Cu^{++} + protein, 1×10^{-5} mM Cu^{++}	Cucumber oxidase	Potato oxidase	Cucumber oxidase	Potato oxidase
No inhibitor	110	110	110	0	0	135
	Per cent inhibition					
Diethyldithiocarbamate, 8×10^{-3} mM*	100	100	100			100
K ethyl xanthate, 0.01 mM	100	100	100			100
NaCN, 0.02 mM	100	100	100			100
NaN_3, 4×10^{-3} mM	100	100	100			100
Thiourea, 0.17 mM	100	100	100			75 (0.3 mM)
Salicylaldoxime, 0.07 mM	100	100	100			60 (0.14 mM)

* The quantities of copper, ascorbic acid, and inhibitors given in the table represent the total amounts in 3.4 ml. of reactants.

TABLE II

Effect of Copper Inhibitors

	Rate of O_2 consumption, c.mm. O_2 per hr. (pH 5.9, 37.2°)						
	Ascorbic acid, 0.01 mM				*d*-Isoascorbic acid, 0.01 mM		
	2×10^{-4} mM Cu^{++}	6×10^{-3} mM Cu^{++} + protein	1×10^{-4} mM Cu^{++} + protein	Cucumber oxidase	2×10^{-4} mM Cu^{++}	1×10^{-4} mM Cu^{++} + protein	Cucumber oxidase
No inhibitor	430	360	360	370	460	380	370
	Per cent inhibition						
Diethyldithiocarbamate, 8×10^{-3} mM*	100	100	100	100	100	100	100
Potassium ethyl xanthate, 0.04 mM	100	100	100	100	100	100	100
Sodium cyanide, 0.04 mM	100	100	100	100	100	100	100
Sodium azide, 0.01 mM	99	98	99	99	99	98	99
Salicylaldoxime, 0.17 mM	100	65	75	65	100	75	65
Thiourea, 0.3 mM	100	90	92	90	100	90	90

* The quantities of copper, ascorbic acid, *d*-isoascorbic acid, and inhibitors in this table represent the total amounts in 3.4 ml. The quantity of albumin in 3.4 ml. was 8 mg. (second column). The total amount of protein in the copper plus cucumber protein was approximately 4 mg. (third and sixth columns).

TABLE III

Effect of Dialysis upon Oxygen Uptake after Inhibition

Temperature 37.2°, pH 5.9 ± 0.1

Inhibitor used*	Ascorbic acid, 0.01 mM, + cucumber oxidase		Catechol, 7×10^{-3} mM, + potato oxidase	
	Inhibited	After dialysis	Inhibited	After dialysis
	c.mm. per hr.	*c.mm. per hr.*	*c.mm. per hr.*	*c.mm. per hr.*
None	370 (initial)	365	135 (initial)	133
NaCN	0	4	0	132
Na diethyldithiocarbamate	0	360	0	130
Thiourea	25	350	30	130

* The concentrations of inhibitors were the same as in Table I.

Snow and Zilva (8) have reported that dialysis destroys the capacity of cucumber juice to serve as a catalyst for the oxidation of dihydroxymaleic acid, apparently by the removal of a quinone carrier.

The copper-protein catalyst formed by adding copper sulfate to an inactivated cucumber protein was specific in promoting the oxidation of *l*-ascorbic and *d*-isoascorbic acids, but it did not affect the oxidation of catechol. It was also found to be irreversibly inactivated when subjected to sodium cyanide inhibition, followed by dialysis.

Each value given in Tables I to III and Fig. 1 represents an average of a number of determinations that were in close agreement.

SUMMARY

1. The rates of oxidation of *l*-ascorbic and *d*-isoascorbic acids were identical when catalyzed by cucumber oxidase, in agreement with the finding of Johnson and Zilva. With copper ion and synthetic copper-albumin as catalysts, the *d*-isoascorbic acid was oxidized slightly more rapidly than *l*-ascorbic acid.

2. In testing the availability of *l*-ascorbic acid and catechol as substrates, it was found that the former, but not the latter, was oxidized in the presence of cucumber oxidase. With potato oxidase, the reverse was true—catechol was oxidized rapidly but ascorbic acid was not affected.

3. Copper ion and synthetic copper-albumin (egg albumin) were practically non-effective as catalysts for the aerobic oxidation of catechol, but both were effective in the presence of *l*-ascorbic and *d*-isoascorbic acids.

4. Typical inhibitors of copper catalysis, thiourea, ethyl xanthate, diethyldithiocarbamate, cyanide, azide, and salicylaldoxime were effective in poisoning both cucumber oxidase and potato oxidase, in comparable concentration ranges.

5. The thiourea and diethyldithiocarbamate inhibitions were readily reversible with both potato and cucumber oxidases, but cyanide inhibition with the latter enzyme was not easily reversed by dialysis.

6. Addition of copper ion to the inactivated cucumber oxidase

restored its activity toward ascorbic acid as a substrate, without showing an increased activity toward catechol.

BIBLIOGRAPHY

1. Stotz, E., Harrer, C. J., and King, C. G., *J. Biol. Chem.*, **119**, 511 (1937).
2. Silverblatt, E., and King, C. G., *Enzymologia*, **5**, 222 (1938).
3. Kubowitz, F., *Biochem. Z.*, **292**, 221 (1937).
4. Kubowitz, F., *Biochem. Z.*, **299**, 32 (1938).
5. Johnson, S. W., and Zilva, S. S., *Biochem. J.*, **31**, 1366 (1937).
6. Rosenberg, H., *Skand. Arch. Physiol.*, **76**, 119 (1937).
7. Tauber, H., Kleiner, I. S., and Mishkind, D., *J. Biol. Chem.*, **110**, 211 (1935).
8. Snow, G. A., and Zilva, S. S., *Biochem. J.*, **32**, 1926 (1938).

STUDIES ON ACETONE-GLYCERALDEHYDE*

IV. PREPARATION OF *d*(+)-ACETONE GLYCEROL

BY ERICH BAER AND HERMANN O. L. FISCHER

(*From the Department of Chemistry, Banting Institute, University of Toronto, Toronto, Canada*)

(Received for publication, December 7, 1938)

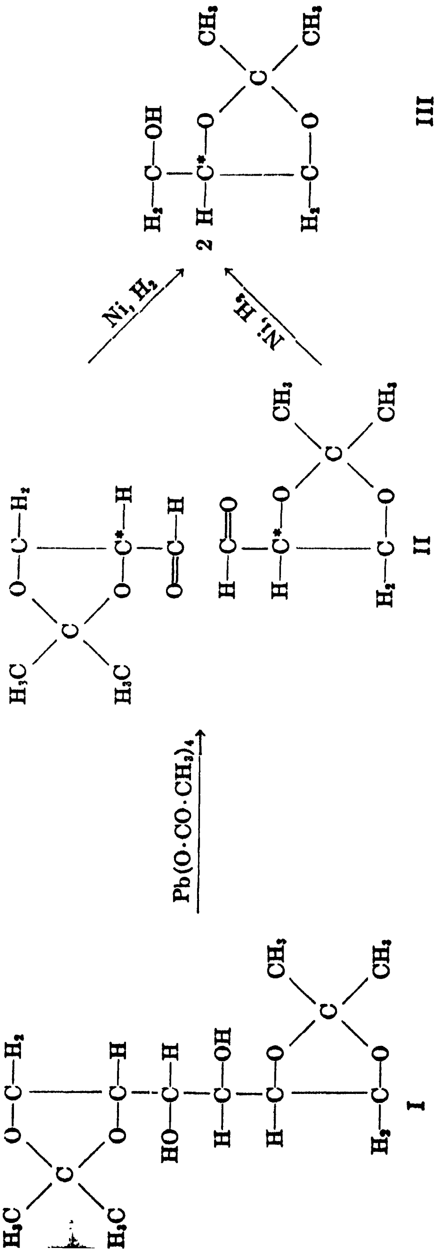
In this communication is described the preparation of *d*(+)-acetone glycerol[1] by catalytic reduction of acetone *d*-glyceraldehyde. We obtained the latter some years ago by the cleavage of 1,2,5,6-diacetone *d*-mannitol (1) with lead tetraacetate (2). Since then we have made several observations as a result of which we have been able to simplify the preparations of diacetone *d*-mannitol (I) and acetone *d*-glyceraldehyde (II), to improve their yields, and thereby to render *d*(+)-acetone glycerol easily obtainable.

In the preparation of 1,2,5,6-diacetone *d*-mannitol (2–5) a notable improvement of the yield, to 55 per cent of the theoretical amount, has now been secured by the use of zinc chloride (6–8), and optimal conditions for the reactions have been established. The isolation of the acetone *d*-glyceraldehyde formed by the oxidative cleavage of diacetone *d*-mannitol by means of lead tetraacetate has been simplified. The esterification of the acetic

---

* The previous communications on acetone-glyceraldehyde have been published in *Berichte der deutschen chemischen Gesellschaft* and *Helvetica chimica acta*, the most recent one (Paper III) 2 years ago (1).

[1] In accordance with the generally accepted usage we designate the spatial relationships of a molecule by the prefixes *d*- and *l*-, and the rotation by (+) and (−). For instance the positively rotating alcohol derived from acetone *d*-glyceraldehyde by reduction we call *d*(+)-acetone glycerol. The position of the prefix before the whole name indicates that the asymmetry is a characteristic only of the substitution compound and not of the glycerol itself. In the special case of acetone glycerol, the rotation has to be determined on the oily substance itself, or on its solution in water-free solvents, because in the presence of water the rotation is decreased and even reversed in sign.


I
Pb(O·CO·CH$_3$)$_4$
II
Ni, H$_2$
Ni, H$_2$
2
III

acid liberated during the reaction has proved unnecessary; it suffices merely to distil off the benzene and the acetic acid through a suitable column, when a yield of nearly 80 per cent of the theoretical is obtained on distilling the residue under reduced pressure. Hydrolysis by acetic acid does not occur in benzene solution.

The above improvements have made easily available a *key substance* useful in the synthesis of the optically active forms of a large number of physiologically important compounds: (1) The product of its acid hydrolysis, *d*-glyceraldehyde,[2] has become important for the synthesis of sugars. (2) Its reduction product, the optically active *d*(+)-acetone glycerol, is the starting point for the preparation of optically active glycerides (10), glycerophosphates, and phosphatides. The reduction of the aldehyde was accomplished in moist ethyl acetate under 80 atmospheres pressure of hydrogen with the nickel catalyst of Rupe, Akermann, and Takagi (11), or with the Raney nickel catalyst, whereby a good yield of *d*(+)-acetone glycerol (III) is secured. A further simplification of the preparation of *d*(+)-acetone glycerol is made possible by conducting the oxidative cleavage of the diacetone *d*-mannitol in ethyl acetate solution. After the removal of the lead diacetate, the dissolved acetone *d*-glyceraldehyde can be immediately reduced with nickel and hydrogen.

The structural uniformity of the *d*(+)-acetone glycerol and its constitution as the 1,2 derivative were established by hydrolysis of its monomethyl ether and titration of the resulting glycerol α-monomethyl ether with periodic acid. The optical rotation of the *d*(+)-acetone glycerol was unaltered by successive benzoylation and saponification, and was numerically of the same degree as that of *l*(−)-acetone glycerol prepared from *l*-arabinose. The asymmetry of the acetone *d*-glyceraldehyde is of course maintained in the glycerol series entirely by the initial presence of the isopropylidene group in the asymmetrical 1,2 position. Should at any stage in the operations the molecule become symmetrical, a subsequent asymmetrical substitution would yield a racemic compound.

---

[2] Pure *d*-glyceraldehyde for use in physiological studies must be free from lead and other heavy metals and should be prepared from distilled acetone *d*-glyceraldehyde by 12 hour hydrolysis in dilute acetic acid at room temperature, followed by concentration *in vacuo*. For chemical purposes it is sufficient to wash the benzene solution of the acetone *d*-glyceraldehyde repeatedly with dilute acetic acid and to concentrate the resulting solutions *in vacuo* ((1) p. 524). By the use of the improvements described here *d*-glyceraldehyde becomes more accessible than the *dl*-glyceraldehyde obtainable by the procedure of Wohl and Neuberg (9).

As we have shown in a preliminary note (10) for the case of the change of *d*(+)-acetone glycerol into *l*(−)-acetone glycerol, it is possible, if the asymmetry is maintained in every step of the reaction, to change active forms into their antipodes. A second example is the conversion of the *d*- and the *l*-isopropylmalonamic acids into each other, exhaustively worked out by Fischer and Brauns (12).

In the next two studies we shall report on the use of this new optically active material for the synthesis of optically active glycerides, and the biological *l*(−)-α-glycerophosphoric acid.

EXPERIMENTAL

1,2,5,6-Diacetone d-Mannitol

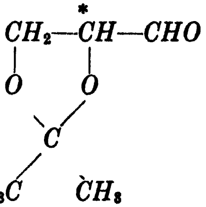
To a solution of 60 gm. of zinc chloride in 300 cc. of acetone, freed of insoluble zinc salts by decantation, 10 gm. of finely powdered *d*-mannitol were added. The mixture was shaken at 20° until solution was complete (2 to 3 hours), allowed to stand for 16 hours,[3] and poured into a solution of 70 gm. of potassium carbonate in 70 cc. of water covered by 300 cc. of ether.[4] The whole was immediately shaken vigorously for half an hour. The acetone-ether solution was poured off the agglomerated pellets of zinc carbonate, the latter were washed twice with 100 cc. of 1:1 acetone-ether solution, and the united filtrates were concentrated in the vacuum of a water pump. The concentrate was heated for 2 hours *in vacuo* at 60–70°. The residue, now completely dry, was five times vigorously refluxed for 20 minutes each in 250 cc. portions of petroleum ether (b.p. 60–80°). The resulting solutions were rapidly filtered through a steam-heated funnel. On slow cooling long, fine crystals of 1,2,5,6-diacetone *d*-mannitol separated; these were filtered off with suction after several hours in the refrigerator. Yield 7.9 gm. (54.8 per cent of the theoretical). The melting point, 119°, could be raised to 122° by recrystallization from water, but for present purposes the product as obtained is sufficiently pure. The residue obtained by concentrating the petroleum ether mother liquor, when dissolved in alcohol and reprecipitated with water, yields fine crystals of triacetone *d*-mannitol (m.p. 68–70°).

---

[3] The preparation of diacetone mannitol can be considerably shortened if the acetone solution is refluxed for a few minutes. However, the mixture should not boil for more than about 5 minutes, otherwise the yield falls to zero, probably owing to the formation of triacetone mannitol. We therefore recommend working at room temperature.

[4] If the acetone solution is allowed to stand, or if it is evaporated *before* removing the zinc chloride, as described in a previous paper (6), triacetone mannitol is produced in a yield of 70 per cent, instead of the desired diacetone mannitol.

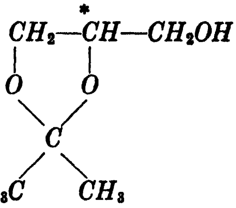
Acetone d-Glyceraldehyde,



$$\underset{\underset{\underset{H_3C\qquad CH_3}{C}}{O\qquad O}}{CH_2-\overset{*}{C}H-CHO}$$

A suspension of 15.6 gm. of 1,2,5,6-diacetone *d*-mannitol in 550 cc. of completely dry, thiophene-free benzene was quickly mixed by gentle stirring or gentle shaking with 26.4 gm. of lead tetraacetate. The sticky precipitate of plumbous salt was triturated to a fine and colorless powder. If, when tested with potassium iodide-starch paper, lead tetraacetate was shown still to be present, diacetone *d*-mannitol was carefully added until all the oxidizing agent was used up. The preparation was filtered and the filtrate carefully concentrated as completely as possible with the use of an efficient fractionating column. The distillate contained, in addition to benzene, traces of acetone *d*-glyceraldehyde and up to 85 per cent of the acetic acid formed in the reaction. The residue, which was usually somewhat cloudy with traces of lead salt and possessed a characteristic odor, was run into a flask with a sealed-on receiver, and the rest of the benzene was evaporated in the vacuum of a water pump. The residual impure acetone *d*-glyceraldehyde distilled at 35–42° under 8 to 11 mm. pressure (bath temperature 60–80°). In several experiments about 12.3 gm. (79.3 per cent of the theoretical) of pure acetone *d*-glyceraldehyde were obtained. In all the above operations the materials and vessels were kept as free as possible from moisture. The further treatment of the acetone *d*-glyceraldehyde must be begun immediately because of its tendency to polymerize.

d(+)-*Acetone Glycerol,*



$$\underset{O-C(CH_3)_2-O}{\underbrace{CH_2-\overset{*}{C}H}}-CH_2OH$$

A solution of 11 gm. of freshly distilled acetone *d*-glyceraldehyde in 90 cc. of ethyl acetate saturated with water was mixed under nitrogen with 10 gm. of nickel catalyst prepared according to Rupe, Akermann, and Takagi (11), and at an initial pressure of 80 atmospheres of hydrogen the mixture was reduced in a rotating autoclave with a glass container for 24 hours at room temperature. The end-point of the reduction is recognized by taking a sample of the solution, distilling off the ethyl acetate, and saponifying the residue in very dilute mineral acid. When, after the residue is made alkaline, no reduction of Fehling's solution is obtained even on heating, the reaction is finished. If the ethyl acetate solution still contained traces of acetone *d*-glyceraldehyde, either more catalyst was added and the reaction continued, or the solution was allowed to stand over potassium carbonate till on acid saponification it no longer gave any reduction of Fehling's solution.

When reduction was complete, the catalyst was filtered off and washed several times with ethyl acetate. The entire filtrate was dried with potassium carbonate in two portions. The dried ethyl acetate solution was concentrated in a fractionating column with glass beads, and the residue fractionally distilled in the vacuum of a water pump. With a short Widmer spiral, even at the first distillation, 9.16 gm. (82 per cent of the theoretical) of pure *d*(+)-acetone glycerol were obtained. Its boiling point under a pressure of 11 to 12 mm. was 80–80.5°; the refractive index n_D was 1.4345 at 19° and 1.4340 at 21°. The same values were observed for the optically inactive acetone glycerol which had been similarly dried in ethyl acetate by potassium carbonate and distilled in the same apparatus. *d*(+)-Acetone glycerol is readily soluble in water, acetone, alcohol, ethyl acetate, chloroform, and benzene, but dissolves in petroleum ether only with difficulty.

Substitution of the Raney nickel catalyst for that of Rupe gave similar results. In this case, with 11 gm. of the Raney nickel-aluminum catalyst, 6.9 gm. of *freshly* prepared acetone *d*-glyceraldehyde dissolved in 80 cc. of ethyl acetate were subjected for 16 hours to 80 atmospheres of hydrogen. 5.3 gm. (75.8 per cent of the theoretical) of *d*(+)-acetone glycerol[5] were obtained. B.p. (11 mm.) = 78–78.5°, $n_D^{25} = 1.4332$.

Titration According to Willstätter and Schudel—In order to obtain a quantitative figure for any traces of acetone *d*-glyceraldehyde remaining in the preparation, a titration with alkaline hypoiodite solution by the method of Willstätter and Schudel (13) was carried out. After 30 minutes none of the iodine solution was used up. Therefore the preparation was absolutely free from acetone *d*-glyceraldehyde.

$C_6H_{12}O_3$ (132). Calculated. C 54.5, H 9.1, acetone 43.93
Found. " 54.5, " 9.0, " 44.35[6]

Optical Rotation—(1) Homogeneous substance in a 1 dm. tube, $\alpha_D^{20} = +13.62°$, $d_4^{20.5} = 1.0704$, $[\alpha]_D^{20} = +12.6°$; (2) in benzene, 0.3038 gm. of substance in 2 cc., 1 dm. tube, $c = 15.19$, $\alpha_D = +1.65°$, $[\alpha]_D = +10.8°$; (3) in water, 0.2582 gm. in 2 cc., 1 dm. tube, $c = 12.91$, $\alpha_{5460.7} = -0.22°$, $[\alpha]_{5460.7} = -1.70°$, $\alpha_D = -0.21°$, $[\alpha]_D = -1.62°$, $\alpha_{6563} = -0.22°$, $[\alpha]_{6563} = -1.70°$, $\alpha_{5105.6} = -0.22°$, $[\alpha]_{5105.6} = -1.70°$; (4) in pyridine, 0.2455 gm. in 2 cc. of absolute pyridine, 1 dm. tube, $c = 12.27$, $\alpha_D = +1.36°$, $[\alpha]_D = +11.09°$; (5) in methyl alcohol, 0.2646 gm. in 2 cc., 1 dm. tube, $c = 13.23$, $\alpha_D = +1.42°$, $[\alpha]_D = +10.7°$.

Molar Refraction—$n_D^{20} = 1.4345$, $d_4^{20} = 1.0704$. Molar refraction calculated, 32.28;[7] found, 32.15.

d(+)-*Acetone Glycerol* (*Simplified Preparation*)—To a cooled solution of 10.4 gm. of diacetone *d*-mannitol in 90 cc. of ethyl acetate, 17.6 gm. of finely powdered lead tetraacetate were added in several portions. Oxidation took place immediately and with a certain development of heat. The precipitate of plumbous salt, at first yellow and gummy, rapidly solidified on agitation. After 15 minutes the preparation was filtered; to the filtrate, which contained only traces of lead salt, 3 cc. of water and 10 gm. of fresh Rupe's nickel catalyst[8] were added, and the mixture was subjected to 80 atmospheres of hydrogen for 24 hours. The further treatment of the reduced solution was carried out in exactly the same way as described in the previous experiment.

---

[5] For the elimination of possible traces of acetone *d*-glyceraldehyde use the method employed in the preceding preparation of *d*(+)-acetone glycerol.

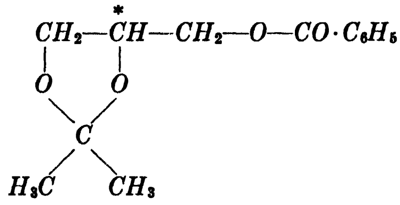
[6] By the method of Messinger (14).

[7] All oxygen was assumed to be hydroxyl oxygen (15).

7.5 gm. (72.8 per cent of the theoretical) of analytically pure *d*(+)-acetone glycerol were obtained. B.p. (10 mm.) = 77–77.5°, refractive index, n_D^{20} = 1.4347.

Optical Rotation—Homogeneous substance in a 1 dm. tube, $\alpha_D = +14.97°$, $d_4^{20.5} = 1.0704$, $[\alpha]_D^{20} = +13.98°$.

(+)-*α-Benzoyl α′,β-Acetone Glycerol*[9] (*from d*(+)-*Acetone Glycerol*)



To a mixture of 3.0 gm. of *d*(+)-acetone glycerol ($[\alpha]_D = +12.6°$) and 3 gm. of absolutely dry quinoline, 3.17 gm. of benzoyl chloride in several portions were added with occasional cooling. After 20 hours water was added and the oil taken up with ether. The ether solution was first washed with potassium carbonate solution for 15 minutes, then rapidly with ice-cold 0.1 N sulfuric acid, and finally with water, dried with potassium carbonate, and concentrated under reduced pressure. The residual oil was twice fractionally distilled in the vacuum of a water pump at a bath temperature of 170–180°. At 10.5 mm., and 159–160.5°, 4.1 gm. (76.4 per cent of the theoretical) of a clear, colorless, viscous distillate were obtained.

$C_{13}H_{16}O_4$ (236.1). Calculated. C 66.1, H 6.8, acetone 24.58, saponification No. 237.2

Found. C 66.1, H 6.8, acetone 24.05, saponification No. 239.5

Refractive Index—$n_D^{17} = 1.5050$, $n_D^{20.5} = 1.5040$.

Optical Rotation—Homogeneous substance in a 1 dm. tube, $d_4^{18} = 1.125$, $\alpha_D = +13.86°$, $[\alpha]_D^{18} = +12.31°$.

---

[8] Raney's nickel catalyst might also be used here.

[9] Prepared according to the directions given by Fischer, Bergmann, and Bärwind for the racemic α-benzoyl acetone glycerol (16).

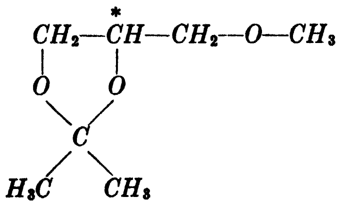
Recovery of d(+)-Acetone Glycerol from (+)-α-Benzoyl α′,β-Acetone Glycerol

To a solution of 2.9 gm. of α-benzoyl α′,β-acetone glycerol in 10 cc. of 96 per cent ethyl alcohol were added 2.5 cc. of 10 N NaOH, and the mixture was set aside at room temperature. After approximately 1½ hours the preparation had set to a gelatinous solid. 6 hours later this was redissolved by the addition of 2.5 cc. of water and left overnight. Then 100 cc. of ether and 5 cc. of water were added, and the whole was thoroughly shaken. The lower aqueous layer was shaken twice with 30 cc. of ether and the united ether extract dried with sodium sulfate. The ether was removed with the help of a fractionating column with glass beads and the residue twice fractionally distilled. At a pressure of 11 mm., bath temperature of 95–100°, 1.25 gm. (77.2 per cent of the theoretical) of analytically pure *d*(+)-acetone glycerol came over at 78.5–79°. $n_D^{20.5} = 1.4347$.

Optical Rotation—Homogeneous substance in a 1 dm. tube, $d_4^{20.5} = 1.0704$, $\alpha_D = +13.54°$, $[\alpha]_D = +12.6°$.

This saponification product is identical with the *d*(+)-acetone glycerol used in the preparation of the α-benzoyl α′,β-acetone glycerol, and its specific rotation is not due to some impurity. The addition of concentrated NaOH to an alcoholic solution of *d*(+)-acetone glycerol does not change its optical rotation during 24 hours at room temperature.

(+)-α-Methyl α′,β-Acetone Glycerol (from d(+)-Acetone Glycerol)



A mixture of 2 gm. of *d*(+)-acetone glycerol, 22 gm. of methyl iodide, and 9 gm. of silver oxide was boiled for 12 hours under a reflux condenser. It was then diluted with absolute ether and filtered with suction. The silver oxide was extracted several times with boiling ether. The united filtrates were dried with sodium sulfate, and the solvent was distilled off through a column. The residue was distilled under reduced pressure, with the bath temperature 65–75°, at 45–49° (10.5 mm.). 1.5 gm. (67.8 per cent of the theoretical) of a clear, mobile liquid possessing a phorone-like odor were obtained. The distillate was distilled once again and separated into two fractions: (I) 10.5 mm., bath 60–65°, vapor 43–44°, 0.7 gm., $n_D^{20.5} = 1.4122$; (II) 10.5 mm., bath 60–65°, vapor 44–46.5°, 0.55 gm. $n_D^{19.5} = 1.4125$.

Fraction I was analyzed:

$C_7H_{14}O_3$ (146). Calculated. C 57.5, H 9.6, acetone 39.75
Found. " 57.3, " 9.5, " 40.10

Optical Rotation—(1) Homogeneous substance in a 1 dm. tube, $d_4^{20.5} = 0.9802$, $\alpha_D = +19.74°$, $[\alpha]_D^{20} = +20.14°$; (2) in pyridine, 45.9 mg. in 0.3 cc. of absolute pyridine, 1 dm. tube, $c = 15.3$, $\alpha_D = +1.97°$, $[\alpha]_D = +12.88°$.

Molar Refraction—$n_D^{20.5} = 1.4122$, $d_4^{20.5} = 0.9802$. Molar refraction calculated, 37.02; found, 37.08.

Titration with Periodic Acid—A solution of 193.7 mg. of the substance in 5 cc. of 0.1 N sulfuric acid was held at room temperature overnight. Then 30 cc. of periodic acid solution containing 394.8 mg. of periodic acid were added, and after 3 hours the excess oxidizing agent was titrated back with arsenious acid, the solution having been made slightly alkaline with bicarbonate. HIO_4 required for pure α-methyl glycerol. Calculated, 254.5 mg.; found 262.3 mg. (103 per cent of the theory).

SUMMARY

From *d*-mannitol optically pure *d*(+)-acetone glycerol with known configuration has been prepared and its constitution proved.

BIBLIOGRAPHY

1. Fischer, H. O. L., and Baer, E., *Helv. chim. acta*, **19**, 519 (1936).
2. Fischer, H. O. L., and Baer, E., *Helv. chim. acta*, **17**, 622 (1934).
3. von Vargha, L., *Ber. chem. Ges.*, **66**, 1394 (1933).
4. Brigl, P., and Gruner, H., *Ber. chem. Ges.*, **67**, 1969 (1934).
5. Fischer, E., and Rund, C., *Ber. chem. Ges.*, **49**, 91 (1916).
6. Fischer, H. O. L., and Taube, C., *Ber. chem. Ges.*, **60**, 485 (1927).
7. Fischer, H. O. L., and Baer, E., *Ber. chem. Ges.*, **63**, 1749 (1930).
8. Fischer, H. O. L., and Dangschat, G., *Ber. chem. Ges.*, **65**, 1038 (1932).
9. Wohl, A., and Neuberg, C., *Ber. chem. Ges.*, **33**, 3099 (1900).
10. Fischer, H. O. L., and Baer, E., *Naturwissenschaften*, **25**, 588, 589 (1937).

11. Rupe, H., Akermann, A., and Takagi, H., *Helv. chim. acta,* **1,** 452 (1918).
12. Fischer, E., and Brauns, F., *Ber. chem. Ges.,* **47,** 3181 (1914).
13. Willstätter, R., and Schudel, G., *Ber. chem. Ges.,* **51,** 780 (1918).
14. Messinger, J., *Ber. chem. Ges.,* **21,** 3366 (1888).
15. Roth, W. A., and Eisenlohr, F., Refraktometrisches Hilfsbuch, Leipzig, 128 (1911).
16. Fischer, E., Bergmann, M., and Bärwind, H., *Ber. chem. Ges.,* **53,** 1592 (1920).

STUDIES ON ACETONE-GLYCERALDEHYDE

V. SYNTHESIS OF OPTICALLY ACTIVE GLYCERIDES FROM *d*(+)-ACETONE GLYCEROL*

By ERICH BAER AND HERMANN O. L. FISCHER

(From the Department of Chemistry, Banting Institute, University of Toronto, Toronto, Canada)

(Received for publication, December 7, 1938)

The use of enantiomorphic forms of optically active glycerides as substrates in biochemical research will presumably throw light on the steric and chemical relationships of a number of interesting physiological transformations (carbohydrate → fat, carbohydrate → phosphatide acids → phosphatides) and on the question of the stereochemical specificity of the enzymes of fat metabolism.[1] Therefore, one of the chief tasks in glyceride synthesis is the preparation of the enantiomorphic forms of asymmetrical types either from optically active material or by resolution of racemic forms, under conditions which should lead to chemically pure products of predetermined constitution and configuration.

In the present paper we describe the preparation of α-monoglycerides and asymmetrically substituted triglycerides containing different acids with the aid of *d*(+)-acetone glycerol.[2] In subsequent papers the preparation of their antipodes from *l*(−)-acetone glycerol will be described.

Of the earlier work on optically active glycerides, we must mention particularly the publications of Abderhalden and Eichwald (4), Bergmann and collaborators (5), Grün and Limpächer (6), and Suzuki and Inoue (7).[3]

---

* An extract of this paper was given before the Sixteenth International Physiological Congress at Zurich, on August 16, 1938, and a preliminary note published (1).

[1] A recent observation of such a relationship will be reported in the following paper (2).

[2] See the preceding article, Paper IV (3).

In these reports, the recorded specific rotations of glycerides are all considerably smaller than those observed by us in comparable cases. The method used by Abderhalden and Eichwald entails in part cyclizations and additions to ethylene oxide rings, reactions in which it is obviously impossible to determine the actual positions of the substituents with desirable accuracy. It is probable that, contrary to the assumption of Abderhalden, the β addition prevails in the addition of acids to the active epihydrin alcohols, and this results in the formation of symmetrical and therefore inactive molecules.[4] In this case the agreement obtained between the rotations of the two series is no guarantee of the structural and optical uniformity of the antipodes. Even the refined methods of Bergmann, Sabetay, Brand, and Dreyer (5) do not yield glycerides with significantly higher rotations. Bergmann therefore recommended a search for methods of resolving the racemic glycerides.

The first to attempt this resolution were Grün and Limpächer (6). The potassium salts of the α,β-diglyceridesulfuric acid esters prepared by them as intermediate products possessed an optical activity abnormal in its magnitude and dependence on temperature, while the diglycerides derived from these showed no perceptible rotation. By a similar process Suzuki and Inoue (7) prepared *d*- and *l*-α-phthalyl-α', β-dibenzoyl glycerol with the relatively high rotations of $[\alpha]_D^{19} = +23.25°$ and $-21.21°$ respectively.

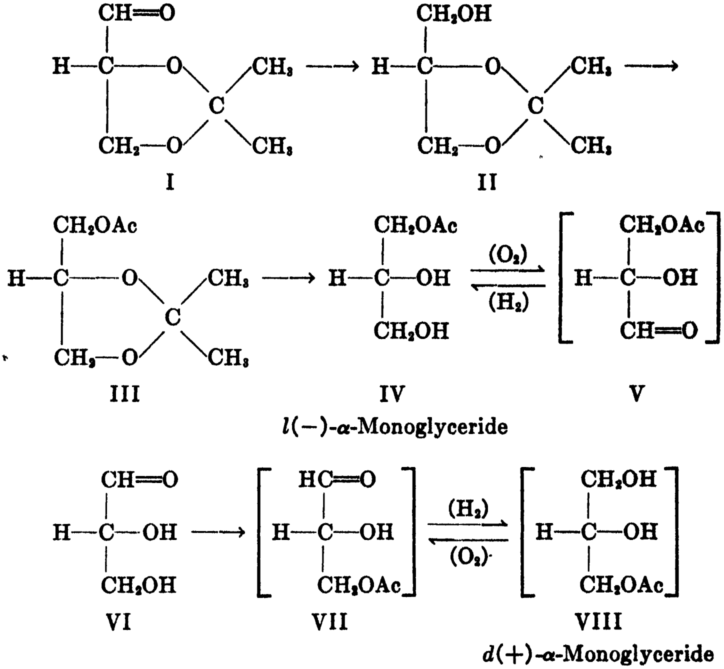
All the foregoing methods of synthesizing enantiomorphic glycerides involve the resolution of glycerol derivatives into their optically active forms with the aid of optically active bases or acids. We have avoided this troublesome procedure by the use of the *d*(+)-acetone glycerol. This choice of material permits at the same time the use of the unambiguous methods worked out by Fischer, Bergmann, and Bärwind (10) for the preparation of inactive glycerides from racemic acetone glycerol. Following

---

[3] An excellent review of previous work in the field of the synthesis of optically active glycerides is given by Grün (8). We shall therefore attempt no more comprehensive review.

[4] In regard to the addition of acid to α-ethylene oxides see Houben (9).

Fischer's directions we have prepared, by acylation of *d*(+)-acetone glycerol with acid chlorides and pyridine or quinoline (see Formulæ II → III), the acetone-α-monoglycerides described below.



α-Acetyl-α′,β-Acetone Glycerol—B.p. (10 to 11 mm.) = 85-86°, n_D^{15} = 1.4288, $[\alpha]_D$ = +3.24° (in homogeneous substance).

α-Lauryl-α′,β-Acetone Glycerol—B.p. (0.002 mm.) = 130-131°, n_D^{20} = 1.4448, $[\alpha]_D^{21}$ = +3.42° (in homogeneous substance).

α-Stearyl-α′,β-Acetone Glycerol—M.p. = 43.5°, α_D^{60} = 3.0-3.5° (in fused substance, 1 dm. tube); $[\alpha]_D$ = +1.9° (in pyridine).

α-Palmityl-α′,β-Acetone Glycerol—M.p. = 33-35°, α_D = +4.38° (in fused substance, 1 dm. tube); $[\alpha]_D$ = +2.48° (in pyridine).

By acid hydrolysis of these acetone compounds we have obtained the corresponding active α-monoglycerides (IV):

α-Monolauryl Glycerol—M.p. = 53-54°, $[\alpha]_D$ = −3.76° (in pyridine).
α-Stearyl Glycerol—M.p. = 76-77°, $[\alpha]_D$ = −3.58° (in pyridine).
α-Palmityl Glycerol—M.p. = 71-72°, $[\alpha]_D$ = −4.37° (in pyridine).

α-(p-Toluenesulfonyl) Glycerol[5]—M.p. = 63–64°, $[\alpha]_D$ = −7.3° (in pyridine).

α-(p-Nitrobenzoyl) Glycerol—M.p. = 88–89°, $[\alpha]_D$ = −17.1° (C_2H_5OH).

In order to test whether in this hydrolysis there is any loss of optical activity due to the migration of the acyl group from the α to the β position, we have availed ourselves of the new titration tests of Criegee (11) and Malaprade (12) for 1,2-glycols. The titration of our monoglycerides according to Criegee showed them to be pure α-glycerides. They are therefore also optically pure. We wish to emphasize that the optically active α-monoglycerides form especially suitable material for investigating acyl migration, so important in the chemistry of fats, because any migration can be ascertained in an unambiguous way both by observing the change in rotation and by using the two methods mentioned above for titration of 1,2-glycols.

With regard to the question of the steric relationships of these α-monoglycerides we believe that the same principle should be followed as that which we have used and briefly explained in our preliminary note ((1) p. 589) on the biologically occurring *l*(−)-α-glycerophosphoric acid.[6] We establish comprehensively the optical classification of the active α-monoglycerides in the following way. According to our preliminary paper the α-monoglycerides would be related to the corresponding *d*- or *l*-glyceraldehyde. Thus an α-monoglyceride which would be formed by the reduction of a 3-acyl-*d*-glyceraldehyde (VII → VIII) is called a *d*-α-monoglyceride, while a glyceride originating from a 3-acyl-*l*-glyceraldehyde is an *l*-α-monoglyceride. In other words, an α-monoglyceride is to be put in the same category with that glyceraldehyde into which it could be transformed by oxidation without any alteration or removal of substituents (IV → V, VIII → VII). This opinion is directly opposed to that of Abderhalden and Eichwald (4) who hold the view that *no* steric classification of the α-monoglycerides is possible, since members of either series may be obtained from the same original material.

---

[5] We give the specific rotation of this compound and the following one, which we will describe in a later paper, in order to show that in the α-monoglycerides replacement of the fatty acid residues by those of aromatic acids considerably increases the rotations observed.

[6] Since we can without constraint consider the α-glycerophosphoric acids as monoglycerides, their coordination is subject to the same points of discussion. We shall therefore in Paper VI (2) refer to these points as definitely proved.

In the preparation of the α-monoglycerides from acetone *d*-glyceraldehyde (I) through *d*(+)-acetone glycerol (II) by acylation of the newly formed hydroxyl group and splitting off the acetone, we have introduced the new substituent into the hydroxyl group of that carbon atom which was part of the carbonyl group of the original acetone *d*-glyceraldehyde. The α-monoglycerides (IV) prepared from *d*(+)-acetone glycerol therefore belong to the *l*-glyceraldehyde series and are to be designated *l*-glycerides, for if one were to oxidize the α-monoglycerides thus obtained, the result, according to the assumptions discussed above, would be 3-acyl-*l*-glyceraldehydes (V). Therefore a change of configuration has taken place, and we find ourselves faced with the remarkable fact that application of the sequence of reactions mentioned above to a derivative of *d*-glyceraldehyde yields a derivative of *l*-glyceraldehyde. This transformation from one steric series into the other one is made possible only by the peculiar kind of asymmetry in the glycerol series, an asymmetry which depends entirely on *substitution.* We have mentioned in the preceding paper two examples of inversion of configuration through a shifting of the substituents. Similarly the corresponding *d*-α-monoglycerides may be synthesized from *l*(−)-acetone glycerol, the preparation of which by reduction of acetone-*l*-glyceraldehyde (13) we have already worked out. We shall report on these at a later date. The biological significance of the above steric classification will be set forth in Paper VI (2).

By further treatment of these α-monoglycerides with acid chlorides and quinoline or pyridine we have prepared the following mixed acid triglycerides:

α-Lauryl-α′,β-Distearyl Glycerol—M.p. = 48.5°, α_D = 0.0° (in pyridine).

α-Stearyl-α′,β-Dipalmityl Glycerol—M.p. = 62.5°, α_D = 0.0° (in chloroform or pyridine).

α-Palmityl-α′,β-Dilauryl Glycerol—M.p. = 44°, α_D = 0.0° (in pyridine).

α-(p-Nitrobenzoyl)-α′,β-Dibenzoyl Glycerol—M.p. = 87–88°, $[\alpha]_D$ = −19.9° (in $C_2H_2Cl_4$).[7]

---

[7] See the preliminary note ((1) p. 588) in which by a typographical error the melting point has been recorded 10° too low.

In order to eliminate the possibility that our original materials—the active α-monoglycerides—had become racemized by the migration of the acyl group under the influence of the tertiary base (pyridine) used in the above preparations, we have exposed them for some time to the action of pyridine. There was, however, no change in rotation observable and the glycerides, when recovered from the solvent, were shown by titration by the method of Criegee to be pure α-monoglycerides. In spite of this, the triglycerides prepared from them so as to preserve their asymmetry show no rotation whatever in the solvents used and in sodium light.[8] This we must accept as a property of triglycerides with fatty acid residues, because α-(*p*-nitrobenzoyl)-α′,β-dibenzoyl glycerol prepared by the same method from *d*(+)-acetone glycerol possesses the considerable rotation of $[\alpha]_D = -19.9°$, probably because this triglyceride absorbs in the range of visible light by virtue of its aromatic substituents.

An optical classification similar to that which we have suggested for the α-monoglycerides seems to be impossible for the triglycerides.

The lack of observable rotation in our synthetic unsymmetrical triglycerides with at least two different fatty acids leads to the suspicion that the *natural* triglycerides of the same type may show no rotation in sodium light. Therefore the natural triglycerides, though they do not show a rotation, are not necessarily racemic, but might easily occur in either of the two enantiomorphic forms (*cf.* Suzuki (14)).

Since, in contrast to the triglycerides, the α-monoglycerides show a large rotation, it is possible that the slight and gradually disappearing rotation sometimes observed in freshly isolated natural fats and oils (7) is due to the presence of α-monoglycerides. We have recently observed that our synthetic α-monoglycerides, even when crystallized, showed a fall of rotation to one-half or one-third of the original values after standing at room temperature for a year. In contrast to these glycerides containing natural fatty acids the α-(*p*-nitrobenzoyl) glycerol and the α-(*p*-nitrobenzoyl)-α′, β-dibenzoyl glycerol retained their high optical activity during the same period, thus proving that no migration of aromatic acyls had taken place. In agreement with our findings, Jackson and King (15) observed no acyl migration in glycerides containing aromatic acid radicals. We are now investigating the migration of the acyls in α-monoglycerides under the influence of heat and alkali by comparing the rate of decrease of optical rotation with the results of the α-glycol titration of Criegee.

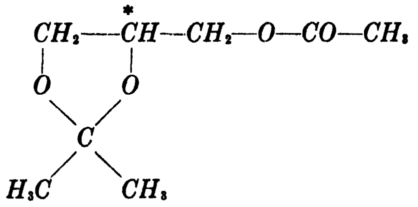
---

[8] We are at present investigating the rotation of these triglycerides in light of different wave-lengths.

A natural compound, the optical activity of which is unquestionably due to the asymmetry of the glycerol part of the molecule, is α-glycerophosphoric acid. It has already been shown that only one of the enantiomorphic forms of this substance is biologically utilized (16). Its synthesis and the proof of its configuration are reported in Paper VI (2).

EXPERIMENTAL

(+)-*α-Acetyl-α′, β-Acetone Glycerol (from d*(+)-*Acetone Glycerol)*



A cooled mixture of 2.64 gm. of *d*(+)-acetone glycerol, 2.0 cc. of pyridine, and 2.0 cc. of acetic anhydride was set aside at room temperature for 16 hours. By two fractional distillations 2.9 gm. (83.3 per cent of the theoretical) of analytically pure (+)-α-acetyl-α′, β-acetone glycerol were secured. B.p. (10 to 11 mm.) = 85–86°, d_4^{20} = 1.0737, n_D^{20} = 1.4280. The corresponding data for the racemic product prepared by Fischer and Pfähler (17) are b.p. (10 to 11 mm.) = 85–86°, d_4^{15} = 1.0770, and n_D^{15} = 1.4288.

$C_8H_{14}O_4$ (174). Calculated. C 55.1, H 8.1, acetone 33.3, saponification No. 322.4

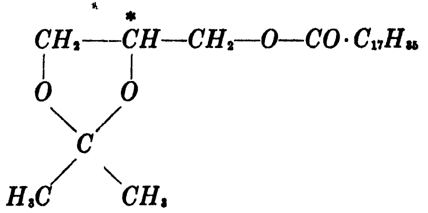
Found. C 54.94, H 8.0, acetone 34.0, saponification No. 330.8

Optical Rotation—(1) Homogeneous substance in a 1 dm. tube, d_4^{20} = 1.0737, α_D^{20} = +3.48°, $[\alpha]_D^{20}$ = +3.24°; (2) in benzene,[9] 0.1981 gm. substance in 2 cc., c = 9.905, 1 dm. tube, α_D = at the most −0.01°, probably 0.00°.

---

[9] The rotation of the other acetonated α-monoglycerides is also zero in benzene.

(+)-*α-Stearyl-α′, β-Acetone Glycerol (from d(+)-Acetone Glycerol)*



To a mixture of 2.6 gm. of *d*(+)-acetone glycerol and 2.7 gm. of absolutely dry quinoline, 6.06 gm. of stearyl chloride were added with constant cooling, and the preparation, which soon solidified, was kneaded thoroughly for several minutes. After 2 days 50 cc. of ether and 50 cc. of 0.5 N sulfuric acid were added, and the ether layer was washed twice with 30 cc. of ice-cold 0.5 N sulfuric acid, twice with 10 cc. of saturated potassium bicarbonate solution, and then with distilled water. The ether solution, dried with sodium sulfate and concentrated, yielded 7.15 gm. (91 per cent of the theoretical) of (+)-α-stearyl-α′, β-acetone glycerol (m.p. 37–38°) which recrystallized once from 150 cc. of boiling 96 per cent alcohol, gave 3.5 gm. of analytically pure glyceride in fine long needles, m.p. 43–43.5° (sinters above 41°). By cooling the mother liquor to 0°, another 2.05 gm. of glyceride were precipitated; this, when recrystallized from 50 cc. of 96 per cent alcohol, also melted at 43°. Total yield of analytically pure substance, 4.6 gm. (58.6 per cent of the theoretical).

$C_{24}H_{46}O_4$ (398.37). Calculated. C 72.3, H 11.6, acetone 14.56, saponification No. 140.5

Found. C 71.8, H 11.4, acetone 15.8, saponification No. 143.2

Optical Rotation—(1) In benzene, 182.9 mg. in 2 cc., $c = 9.14$, 1 dm. tube, $\alpha_D = 0.0°$; (2) in pyridine, 178.4 mg. in 2.02 cc. of absolute pyridine, $c = 8.83$, 1 dm. tube, $\alpha_D = +0.17°$, $[\alpha]_D = +1.9°$; (3) in melted substance, 1 dm. tube, α_D = between +3.0° and +3.5°.

l(−)-*α-Monostearyl Glycerol*

$$C_{17}H_{35}\cdot CO\cdot O\cdot CH_2\text{—}\overset{*}{C}H(OH)\text{—}CH_2\cdot OH$$

To an ice-cooled solution of 3.3 gm. of (+)-α-stearyl-α′, β-acetone glycerol, in 17 cc. of ether, 17 cc. of concentrated HCl were added in small portions as rapidly as possible, a rise of temperature above 25° being avoided. Soon the solution separated into two layers and the monostearin began to appear. After 30 minutes 70 cc. of water were added, and the preparation was allowed to stand another half hour in the freezing mixture. Then the floating precipitate was sucked off, thoroughly washed with water, and dried over phosphorus pentoxide. Yield, 2.8 gm. (94 per cent of the theoretical). When the material was recrystallized twice from ether, the melting point was raised from 74–75° to 76–77° (sinters above 73°) where it remained constant after further recrystallization.

For analysis the substance which had been recrystallized twice was dried over phosphorus pentoxide in a high vacuum.

$C_{21}H_{42}O_4$. Calculated. C 70.3, H 11.8, saponification No. 156.5
358.3 Found. " 70.4, " 11.8, " " 160.5

Optical Rotation—In pyridine, 246.2 mg. in 2 cc., $c = 12.31$, 1 dm. tube, $\alpha_D = -0.44°$, $[\alpha]_D = -3.58°$.

Titration of Adjacent Hydroxyl Groups—123.8 mg. of the substance were dissolved in 15 cc. of glacial acetic acid, and 15 cc. of glacial acetic acid containing 342.7 mg. of lead tetraacetate were added. After 24 hours, 148 mg. were used up; *i.e.*, 97.0 per cent of the theoretical amount. From the amount of lead tetraacetate used was subtracted the amount which disappeared in the same period in the corresponding control blank. This same deduction was made in all the succeeding cases of titration with lead tetraacetate.

α-Stearyl-α′,β-Dipalmityl Glycerol

$$C_{17}H_{35}\cdot CO\cdot O\cdot CH_2—\overset{*}{C}H(O\cdot CO\cdot C_{15}H_{31})—CH_2\cdot O\cdot CO\cdot C_{15}H_{31}$$

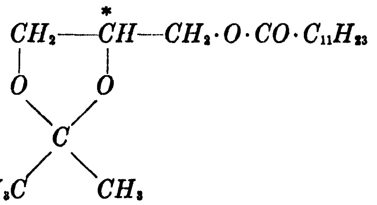
To a solution of 0.75 gm. of *l*(−)-α-stearyl glycerol in 6 cc. of dry chloroform were added 1.15 gm. of palmityl chloride and 0.65 gm. of quinoline dissolved in 1.5 cc. of chloroform, and the preparation was kept at 40° for 24 hours. The oily precipitate formed after some time was brought again into solution by the addition of more chloroform. After dilution with 80 cc. of ether the solution was washed successively with sulfuric acid, potassium carbonate solution, and water, dried with sodium sulfate, and concentrated. Yield, 1.65 gm. (94.4 per cent of the theoretical) of almost pure triglyceride. The melting point of the crude product, 61–62°, could not be raised above 62.5° in spite of repeated recrystallization. The substance dissolves easily in chloroform and pyridine.

For analysis the substance which had been recrystallized several times was dried at room temperature over P_2O_5 in a high vacuum.

$C_{53}H_{102}O_6$ (834.8). Calculated. C 76.2, H 12.3
Found. " 76.0, " 12.3

Optical Rotation—(1) In chloroform, 164.6 mg. in 2 cc. of absolute chloroform, $c = 8.23$, 1 dm. tube, $\alpha_D = \pm 0.0°$; (2) in pyridine, 92.4 mg. in 2 cc., $c = 4.62$, 1 dm. tube, $\alpha_D = \pm 0.0°$.

(+)-*α-Lauryl-α′,β-Acetone Glycerol (from d(+)-Acetone Glycerol)*



The lauryl acetone glycerol was prepared in the same way as the corresponding stearyl compound already described. 1 gm. of *d*(+)-acetone glycerol, 3.2 gm. of quinoline, and 5.26 gm. of lauryl chloride, after treatment in the usual manner, two distillations in a high vacuum, and separation of a first fraction which crystallized in the receiver, yielded 6.0 gm. (81.1 per cent of the theoretical) of a clear colorless liquid. B.p. (0.002 mm.) = 130–131° (bath 150–160°), $n_D^{20} = 1.4448$.

$C_{18}H_{34}O_4$ (314.27). Calculated. C 68.7, H 10.9, acetone 18.45, saponification No. 178.5
Found. C 68.8, H 10.7, acetone 19.2, saponification No. 176.5

Optical Rotation—(1) Homogeneous substance in a 1 dm. tube, 21°, $\alpha_D = +3.42°$; (2) in pyridine, 184.7 mg. in 2 cc. of absolute pyridine, $c = 9.23$, 1 dm. tube, $\alpha_D = +0.11°$, $[\alpha]_D = +1.2°$.

l(−)-*α-Monolauryl Glycerol*

$$C_{11}H_{23}\cdot CO\cdot O\cdot CH_2\text{—}\overset{*}{C}H(OH)\text{—}CH_2OH$$

3.45 gm. of (+)-*α*-lauryl acetone glycerol were hydrolyzed by the procedure given for the *l*(−)-*α*-monostearyl glycerol, with the one difference that the temperature never rose above 0°. Yield, 2.87 gm. (95 per cent of the theoretical) of fairly pure (−)-α-lauryl glycerol. The melting point of 51–52° (sinters above 49°) was raised to 53–54° by two recrystallizations from petroleum ether (b.p. = 40–60°) and then remained constant on further recrystallization.

For analysis the substance was dried over phosphorus pentoxide in a high vacuum at room temperature.

$C_{15}H_{30}O_4$.	Calculated.	C 65.6,	H 11.0,	saponification No. 204
274.24	Found.	" 65.95,	" 11.12,	" " 209.5

Optical Rotation—In pyridine, 197.1 mg. of absolutely dry pyridine made up to 2 cc., c = 9.85, 1 dm. tube, α_D = −0.36°, $[\alpha]_D$ = −3.7°.

Titration of 1,2-Glycol with Lead Tetraacetate—To a solution of 113.4 mg. of α-lauryl glycerol in 10 cc. of glacial acetic acid, 10 cc. of a glacial acetic acid solution containing 274.3 mg. of lead tetraacetate were added. After 48 hours the excess lead tetraacetate was determined by back titration. Calculated, lead tetraacetate used up, 183.2 mg.; found, lead tetraacetate used up, 167.7 mg. (91.5 per cent of the theoretical).

α-Lauryl-α′,β-Distearyl Glycerol

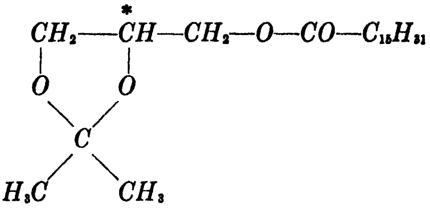
$$C_{11}H_{23}\cdot COOCH_2—\overset{*}{C}H(O\cdot CO\cdot C_{17}H_{35})—CH_2\cdot O\cdot CO\cdot C_{17}H_{35}$$

To a solution of 1.22 gm. of l(−)-α-lauryl glycerol in 1.5 cc. of dry chloroform, a solution of 2.69 gm. of stearyl chloride and 1.2 cc. of quinoline in 3.00 cc. of chloroform was added while cooled with ice. The mixture, which soon solidified by the precipitation of quinoline hydrochloride, was set aside for 2 days at room temperature. After the addition of 30 cc. of 0.5 N sulfuric acid and 120 cc. of ether with vigorous shaking, the ether layer was taken off and washed successively with dilute sulfuric acid, potassium carbonate solution, and water. The ether solution, dried with sodium sulfate, concentrated to 30 cc., and cooled to −10°, yielded 3.0 gm. (83 per cent of the theoretical) of triglyceride. M.p. = 45–46° (crude material). After three recrystallizations from alcohol-ether (1:1) the melting point rose to 48.5°.

$C_{51}H_{98}O_6$ (806.78).	Calculated.	C 75.86,	H 12.24
	Found.	" 75.79,	" 12.1

Optical Rotation—In pyridine, 199 mg. in 2 cc., c = 9.95, 1 dm. tube, α_D = 0.0°.

(+)-α-Palmityl-α′,β-Acetone Glycerol



This palmityl compound was prepared in the same way as the corresponding stearyl compound already described. From 2.6 gm. of d(+)-acetone glycerol, 2.7 gm. of quinoline, and 5.48 gm. of palmityl chloride, 6.52 gm. (89 per cent of the theoretical) of α-palmityl-α′,β-acetone glycerol were obtained with a melting point of 31–34° with sintering above 26°. After the crude product (6.5 gm.) was dissolved in 25 cc. of boiling 96 per cent ethyl alcohol and cooled slowly to room temperature, 3.5 gm. of analytically pure α-palmityl acetone glycerol were recovered in long crystals with a melting point of 33–35°.

For analysis the substance was dried over P_2O_5 in a high vacuum at room temperature.

$C_{22}H_{42}O_4$ (370.34). Calculated. C 71.3, H 11.4, acetone 15.7, saponification No. 151.2
Found. C 71.6, H 11.2, acetone 15.8, saponification No. 145.5

Optical Rotation—(1) In benzene, 281.5 mg. in 2 cc., 1 dm. tube, $\alpha_D = 0.0°$; (2) in pyridine, 185.6 mg. in 2 cc., $c = 9.28$, 1 dm. tube, $\alpha_D = +0.23°$, $[\alpha]_D = +2.5°$; (3) in melted substance, temperature 50–52°, 1 dm. tube, $\alpha_D = +4.38°$.

l(−)-α-Palmityl Glycerol

$$C_{15}H_{31}\cdot CO\cdot O\cdot CH_2—\overset{*}{C}H(OH)—CH_2(OH)$$

2.45 gm. of (+)-α-palmityl-α′,β-acetone glycerol were hydrolyzed by the preceding process. Yield, 2.1 gm. (96 per cent of the theoretical) of crude product. The melting point of the substance was raised, by twice recrystallizing from boiling ether, from 69–70° to 71–72°, and remained there on further recrystallization.

For analysis the recrystallized substance was dried over phosphorus pentoxide in a high vacuum.

$C_{19}H_{38}O_4$.	Calculated.	C 69.0, H 11.6, saponification No. 169.4
330.3	Found.	" 69.1, " 11.5, " " 181.4

Optical Rotation—In pyridine, 155.8 mg. in 2 cc., $c = 7.79$, 1 dm. tube, $\alpha_D = -0.34°$, $[\alpha]_D = -4.37°$.

Titration of Adjacent Hydroxyl Groups—To 95.9 mg. of substance dissolved in 15 cc. of glacial acetic acid, was added a solution of 345.3 mg. of lead tetraacetate in 15 cc. of glacial acetic acid. After 22 hours, in comparison with the calculated 128.6 mg. of lead tetraacetate, 124.4 (96.7 per cent) had been used up.

α-Palmityl-α′,β-Dilauryl Glycerol

$$C_{15}H_{31}COOCH_2—\overset{*}{C}H(OCO \cdot C_{11}H_{23})—CH_2 \cdot OCO \cdot C_{11}H_{23}$$

0.55 gm. of (−)-α-palmityl glycerol was dissolved with moderate heating in 2 cc. of absolute chloroform. To the solution were added 0.6 gm. of absolute pyridine and then gradually with cooling 0.72 gm. of lauryl chloride. After 24 hours at 40° the preparation was treated in the usual manner. 1.0 gm. (86.5 per cent of the theoretical) of triglyceride was obtained with a melting point of 42–43°. The melting point could be raised only to 44° by recrystallization from absolute alcohol. The triglyceride crystallizes in small, centrally grouped needles.

$C_{43}H_{82}O_6$ (694.6).	Calculated.	C 74.3, H 11.9
	Found.	" 74.1, " 11.6

Optical Rotation—In pyridine, 104.0 mg. in 2 cc., $c = 5.20$, 1 dm. tube, $\alpha_D = \pm 0.0°$.

Migration of Acyl Radical in α-Monoglycerides under Influence of Pyridine

For assurance regarding the position of the fatty acid residues in synthetic mixed acid triglycerides it was necessary to ascertain whether, under the usual acylation conditions, pyridine causes a significant migration of the acyl group of a monoglyceride from the α to the β position. By observing the rotation of the α-monoglycerides in pyridine, and titrating the α-monoglycerides with lead tetraacetate after recovery from pyridine, it was unequivocally shown that no migration takes place.

	α_D in pyridine immediately after solution	α_D in pyridine after 72 hrs. at 18-20°
	degrees	*degrees*
l(−)-α-Monolauryl glycerol	−0.36	−0.38
l(−)-α-Monopalmityl "	−0.34	−0.34
l(−)-α-Monostearyl "	−0.44	−0.48

Titration, by the method of Criegee, of monoglycerides isolated from pyridine after 72 hours in solution gave the following results. 127.1 mg. of (−)-α-monolauryl glycerol used up 195 mg. of $PbAc_4$ (95 per cent of the calculated 205.3 mg.); 95.9 mg. of (−)-α-monopalmityl glycerol used up 124.4 mg. of $PbAc_4$ (97.9 per cent of the calculated 127 mg.); 123.8 mg. of (−)-α-monostearyl glycerol used up 148.0 mg. of $PbAc_4$ (96.7 per cent of the calculated 153.0 mg.).

SUMMARY

1. Starting from the *d*(+)-acetone glycerol described in Paper IV we have prepared optically active α-monoglycerides.

2. The α-monoglycerides have been sterically related to the active glyceraldehydes.

3. The crystallized α-monoglycerides with fatty acid residues lose a considerable part of their activity over a period of 1 year even when kept under normal conditions. Aromatic α-monoglycerides are much more stable; an analogous migration of the acyls does not occur for instance in the case of *l*(−)-α-*p*-nitrobenzoyl glycerol.

4. Synthetic triglycerides containing different *fatty* acids showed no observable optical activity, but they are believed to be latently optically active, since a mixed triglyceride with *aromatic* acid residues showed a definite rotation.

BIBLIOGRAPHY

1. Fischer, H. O. L., and Baer, E., *Naturwissenschaften*, **25**, 588, 589 (1937).
2. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **128**, 491 (1939).
3. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **128**, 463 (1939).

4. Abderhalden, E., and Eichwald, E., *Ber. chem. Ges.*, **47**, 1858 (1914); **48**, 113, 1851 (1915).
5. Bergmann, M., *Z. physiol. Chem.*, **137**, 27 (1924). Bergmann, M., and Sabetay, S., *Z. physiol. Chem.*, **137**, 47 (1924). Bergmann, M., Brand, E., and Dreyer, F., *Ber. chem. Ges.*, **54**, 936 (1921).
6. Grün, A., and Limpächer, R., *Ber. chem. Ges.*, **60**, 255 (1927).
7. Suzuki, B., and Inoue, Y., *Proc. Imp. Acad. Japan*, **6**, 71 (1930); *Chem. Abst.*, **24**, 4265 (1930); *Chem. Zentr.*, 1930, II, 1063.
8. Grün, A., in Schönfeld, H., Chemie und Technologie der Fette und Fettprodukte, Vienna, **1**, 264 (1936).
9. Houben, J., Die Methoden der organischen Chemie, Leipzig, 3rd edition, **3**, 224 (1930).
10. Fischer, E., Bergmann, M., and Bärwind, H., *Ber. chem. Ges.*, **53**, 1589 (1920).
11. Criegee, R., *Ann. Chem.*, **481**, 263 (1930); *Ber. chem. Ges.*, **64**, 260 (1931).
12. Malaprade, L., *Compt. rend. Acad.*, **186**, 382 (1928).
13. Fischer, H. O. L., and Baer, E., *Science*, **88**, 2274 (1938).
14. Suzuki, B., *Proc. Imp. Acad. Japan*, **7**, 230 (1931); *Chem. Zentr.*, 1931, II, 2345.
15. Jackson, D. T., and King, C. G., *J. Am. Chem. Soc.*, **55**, 678 (1933).
16. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **264**, 62 (1933).
17. Fischer, E., and Pfähler, E., *Ber. chem. Ges.*, **53**, 1606 (1920).

STUDIES ON ACETONE-GLYCERALDEHYDE

VI. SYNTHESIS OF THE BIOLOGICAL $l(-)$-α-GLYCEROPHOSPHORIC ACID*

BY ERICH BAER AND HERMANN O. L. FISCHER

(From the Department of Chemistry, Banting Institute, University of Toronto, Toronto, Canada)

(Received for publication, December 7, 1938)

In Paper V (2) it was demonstrated by the synthesis of optically active α-monoglycerides that asymmetrical α-monoglycerides of both plant and animal origin should also show activity when present only in one of the enantiomorphic forms. A causal relationship between optical activity and the asymmetry of the glycerol molecule in naturally occurring glycerides has not yet been established with complete validity.[1] The chief reason for this appears to be the difficulty of isolating pure substances from natural fats, which consist of nearly inseparable mixtures of many glycerides, without altering the original constitution and configuration in the process. Suzuki and Inoue have found that freshly extracted natural fats are optically active and show a declining rotation ((2) p. 480). Unequivocal proof that this activity results from the asymmetry of the glycerol molecule in natural glycerides has not yet been obtained.

In the case of α-glycerophosphoric acid, which has long been known as a component of the lecithins and cephalins and may in a sense be looked on as a monoglyceride, the situation is much more favorable. Because of its great stability it has been possible for several workers[2] to separate it from various phosphatides and free it from its accompanying β isomer ((5) pp. 5, 10, 11 (6)).

---

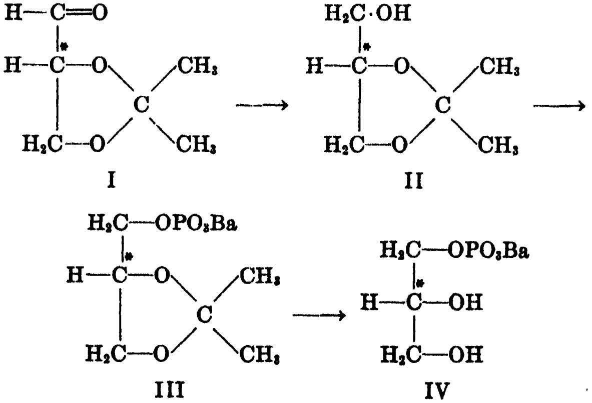
* An extract of this paper was given before the Sixteenth International Physiological Congress at Zurich, on August 16, 1938, and a preliminary note published (1).

[1] See for instance the article of Grün (3).

[2] Thierfelder and Klenk give a comprehensive résumé of the chemistry of the glycerophosphoric acids in their book (4).

By preparing the comparatively highly active dimethyl ether dimethyl ester of α-glycerophosphoric acid from its barium salt, which shows no perceptible rotation, Karrer and Salomon ((5) pp. 10, 11) were able to show conclusively that the α-glycerophosphoric acid in the phosphatides examined by them is optically active and present in only one enantiomorphic form. Meyerhof and Kiessling (7) later established its identity with the glycerophosphoric acid which forms an intermediate stage in alcoholic fermentation and glycolysis, and observed (8) that the biological *l*(−)-α-glycerophosphoric acid is completely utilized by the enzyme of muscle press-juice, while its antipode remains entirely unaltered.

The preparation of the enantiomorphic forms of α-glycerophosphoric acid was undertaken by Abderhalden and Eichwald (9) and by Karrer and Benz (10). However, the steric relationship between the α-glycerophosphoric acids and compounds of known configuration (active glyceraldehydes for example) could not be deduced from any information as yet available. The preparation of the two active α-glycerophosphoric acids was therefore undertaken for the purpose of relating their configuration in the sense of Wohl and Freudenberg to that of the active glyceraldehydes. We describe first of all the synthesis of *l*(−)-α-glycerophosphoric acid.



I II III IV

In this operation we have used the same sequence of reactions as that employed by Fischer and Pfähler (11) in the synthesis of racemic α-glycerophosphoric acid. *d*(+)-Acetone glycerol (II) was phosphorylated with the help of phosphorus oxychloride and quinoline, the acetone-protective group was removed by acid hydrolysis, and the α-glycerophosphoric acid isolated as the barium salt (IV).

By this procedure the point of entrance of the phosphoric acid is determined beforehand and remains unchanged after the removal of the acetone. The conditions of the synthesis are such that a migration of the acyl group which would mean either racemization or a change in configuration is impossible. This can be checked by titration by the method of Malaprade (12). The relationship of the configuration of the α-glycerophosphoric acid obtained to that of the *d*(+)-acetone glycerol, that of the acetone *d*-glyceraldehyde (I), and hence to that of the *d*-glyceraldehyde itself, is easily followed. This relationship, on the basis of the argument used in Paper V of this series for the deduction of the configuration of the α-monoglycerides ((2) p. 479), indicates that the α-glycerophosphoric acid synthesized from *d*(+)-acetone glycerol is to be classed in the *l* series. Its correct designation is therefore *l*(−)-α-glycerophosphoric acid.

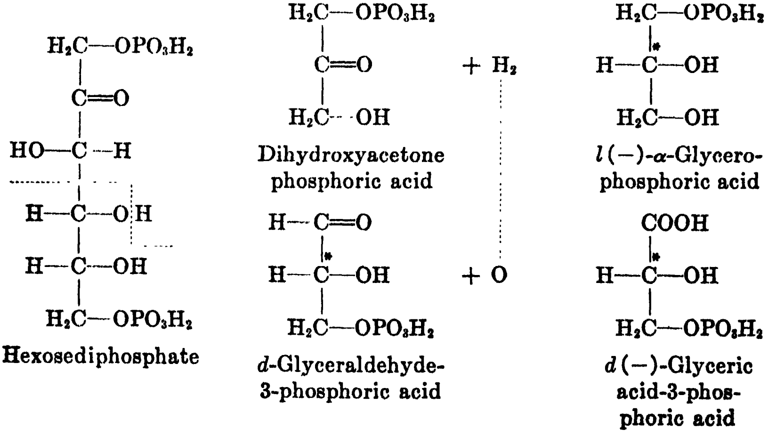
The identity of our synthetic acid with the "biological α-glycerophosphoric acid" from glycolysis, alcoholic fermentation, and from phosphatides was established by observing the rotation of the dimethyl ether dimethyl ester.

Dimethyl Ether of Methyl α-Glycerophosphate—(*a*) Synthetic, $[\alpha]_D = -4.78°$; (*b*) from phosphatides, $[\alpha]_D = -3.2°$ ((5) p. 12); (*c*) from glycolysis and fermentation, $[\alpha]_D^{20} = -4.46°$ (7).

In agreement with the work of Karrer and Salomon the barium salt of the synthetic *l*(−)-α-glycerophosphoric acid showed no perceptible rotation. The weak rotations given in the literature for the barium salt of α-glycerophosphoric acid from phosphatides are therefore to be ascribed to the presence of optically active impurities ((5) p. 9). Further, the silver salt of the synthetic acid shows a specific rotation of only $[\alpha]_D = +1.0°$ (in ammoniacal solution). Kiessling and Schuster (13) found $[\alpha]_D = +0.8°$ (saturated aqueous solution) for the silver salt of the natural α-glycerophosphoric acid from fermentation. The diethyl ester of the diethyl ether, $[\alpha]_D^{20} = -5.73°$, may in future investigations prove to be a valuable preparation for comparison.

Now that synthesis has fixed the steric relationship of the α-glycerophosphoric acids to the glyceraldehydes, we may proceed to draw certain biological conclusions: In the fermentation scheme of Embden, as developed by Meyerhof and his collaborators, the dismutation of triosephosphoric acids plays an important part. Since α-glycerophosphoric acid is the product of simple reduction of triosephosphoric acids, it follows from our purely chemical evidence that the natural *l*(−)-α-glycerophosphoric acid does not arise biologically from the natural *d*-glyceraldehyde-3-phosphoric acid, because the latter belongs to another steric series, but that it must have been formed by asymmetrical fermentative reduction from dihydroxyacetone phosphoric acid.

Our work on the configuration of *l*(−)-α-glycerophosphoric acid, which we described to Professor O. Meyerhof in a personal communication before any of these results were published ((1) p. 589), stimulated his collaborators, Dr. W. Kiessling and Dr. P. Schuster, to ascertain whether the biological α-glycerophosphoric acid isolated from yeast, would, on oxidation by bromine, yield the natural levorotatory glyceric acid-3-phosphoric acid or its antipode. As we expected, they (13) obtained from the biological *l*(−)-α-glycerophosphoric acid the unbiological, dextrorotatory glyceric acid-3-phosphoric acid which belongs to the *l* series. This is further evidence that the two dismutation products of triosephosphoric acids, α-glycerophosphoric acid and glyceric acid-3-phosphoric acid, belong to two different steric series.



Hexosediphosphate

Dihydroxyacetone phosphoric acid

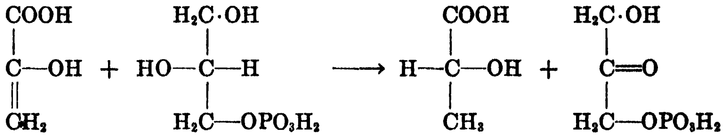
d-Glyceraldehyde-3-phosphoric acid

l(−)-α-Glycerophosphoric acid

d(−)-Glyceric acid-3-phosphoric acid

Thus the natural glyceric acid-3-phosphoric acid must belong to the *d* series and accordingly can arise by oxidation only from *d*-glyceraldehyde-3-phosphoric acid. Summing up these results, we obtain the accompanying scheme for the dismutation of triosephosphoric acids, which we have already presented in our preliminary paper ((1) p. 589).

In the light of this new steric relationship the well known Embden-Meyerhof reaction of pyruvic acid and α-glycerophosphoric acid which has hitherto been assumed to yield lactic acid and *d*-glyceraldehyde-3-phosphoric acid, must now be written thus:



$$\begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{C{-}OH} \\ \| \\ \mathrm{CH_2} \end{array} + \begin{array}{c} \mathrm{H_2C\cdot OH} \\ | \\ \mathrm{HO{-}C{-}H} \\ | \\ \mathrm{H_2C{-}OPO_3H_2} \end{array} \longrightarrow \begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}OH} \\ | \\ \mathrm{CH_3} \end{array} + \begin{array}{c} \mathrm{H_2C\cdot OH} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{H_2C{-}OPO_3H_2} \end{array}$$

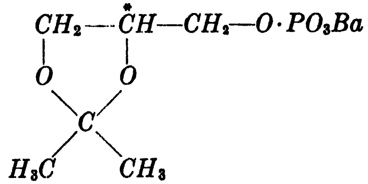
The fact that *l*-glyceraldehyde phosphoric acid is not utilized biologically (as we know from experience with *dl*-glyceraldehyde-3-phosphoric acid) compels the conclusion that dihydroxyacetone phosphoric acid must be formed in this reaction.

By the same series of reactions as for the natural *l*(−)-α-glycerophosphoric acid, but starting from *l*(−)-acetone glycerol, which we obtained from *l*-mannitol, it is easy to prepare the *d*(+)-α-glycerophosphoric acid which has not yet been found in nature. Both acids are ideal starting materials for the synthesis of phosphatide acids and phosphatides of which the configuration is known with regard to the β-carbon atom of the glycerol residue. Since only the *l*(−)-α-glycerophosphoric acid has so far been found in natural substances, we believe that most of the α-phosphatides which have as yet been investigated are derived from this acid, rather than from its antipode.

We consider it probable that an important rôle is played by *l*(−)-α-glycerophosphoric acid in the transformation of carbohydrate into fat in the bodies of higher animals. It seems to be the substance which, formed from phosphorylated sugars, in fermentation and glycolysis, would supply the asymmetry for the synthesis of optically active α-phosphatides and fats in nature.

EXPERIMENTAL

(*I*) *Barium Salt of Acetone Compound of l*(−)-*α-Glycerophosphoric Acid* (*from d*(+)-*Acetone Glycerol*)



The preparation was carried out according to the method of Fischer and Pfähler (11) for the corresponding optically inactive substance. From 3.0 gm. of *d*(+)-acetone glycerol 3.9 gm. of barium salt were obtained. Analysis of the substance, dried in air to constant weight, was as follows:

$C_6H_{11}O_6PBa + 3H_2O$.	Calculated.	C 17.9,	H 4.2,	P 7.7,	acetone 14.4
401.5	Found.	" 18.2,	" 4.3,	"[3] 7.76,	"[4] 14.4

Optical Rotation—0.5928 gm. of barium salt in 20 cc. of water, c = 2.96' 2 dm. tube, $\alpha_D = \pm 0.0°$.

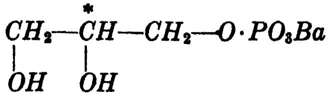
(*II*) *Silver Salt of Acetone Compound of l*(−)-*α-Glycerophosphoric Acid*

0.4 gm. of the above preparation (I) was dissolved in 15 cc. of water. A concentrated solution of 0.35 gm. of silver nitrate was added in the dark. After some time the silver salt was sucked off, washed with a few drops of alcohol and ether, and dried in a high vacuum at room temperature over P_2O_5. Yield, 0.26 gm. (68.5 per cent of the theoretical). The salt was slightly reddish in color.

$C_6H_{11}O_6PAg_2$ (425.9). Calculated, Ag 50.7; found, Ag 50.96

Optical Rotation—229.0 mg. in 2 cc. of pyridine + water (3:1), solution decolorized with animal charcoal, 1 dm. tube, $\alpha_D = +0.29°$ (read after 20 hours), $[\alpha]_D = +2.5°$.

(*III*) *Barium l*(−)-*α-Glycerophosphate* (*from d*(+)-*Acetone Glycerol*)



$$\underset{OH}{CH_2}-\overset{*}{\underset{OH}{CH}}-CH_2-O\cdot PO_3Ba$$

---

[3] Alkalimetric determination of phosphoric acid according to Neumann (14).

[4] Determined according to Messinger (15).

The preparation of this substance was carried out according to the method of Fischer and Pfähler (11). To a solution of 1.3 gm. of the barium salt of acetone α-glycerophosphoric acid (I) in 35 cc. of water, 9.1 cc. of 1 N sulfuric acid were added, and the mixture heated for 20 minutes in a boiling water bath. After cooling, 1.9 gm. of barium hydroxide were added, and the excess was removed with carbon dioxide. The filtrate was evaporated to dryness in the vacuum of a water pump at a bath temperature of 30–35°, dissolved in a little water, filtered, and brought to the boiling point. The precipitated barium salt was sucked off and dried for 30 minutes in a high vacuum over boiling xylene. Yield, 0.65 gm. (65 per cent of the theoretical).

$C_3H_7O_6PBa$ (307.48). Calculated. C 11.7, H 2.3, P 10.08 (14)
Found. " 11.7, " 2.7, " 10.08

Optical Rotation of Free Acid—206.9 mg. of barium salt in 2 cc. of 2 N HCl, 1 dm. tube, $\alpha_D = -0.15°$, $[\alpha]_D = -1.45°$.

Simplified Preparation of Barium l(−)-α-Glycerophosphate (III)—In the preparation of (III) it was found unnecessary to isolate the barium salt of acetone l(−)-α-glycerophosphoric acid (I) in order to split off the acetone residue by hydrolysis.

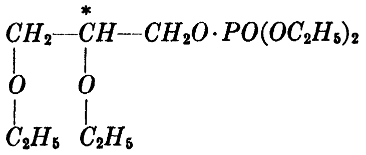
A mixture of 4.1 gm. of d(+)-acetone glycerol and 12 cc. of pure quinoline was dropped during 20 minutes with continuous shaking into a solution of 4.75 gm. of phosphorus oxychloride in 15 cc. of quinoline cooled in a freezing mixture. The whole was kept in the freezing mixture for 1 hour, and for 2 hours at room temperature. The pasty solution was then poured into 350 cc. of ice water to which 15 gm. of concentrated sulfuric acid and 14 gm. of silver carbonate had been added, and was vigorously stirred for 30 minutes. The clear solution was filtered through animal charcoal, and the silver removed with hydrogen sulfide. The strongly acid filtrate was heated for 15 minutes in a boiling water bath. It was then cooled to 0° and 45 to 50 gm. of finely powdered barium hydroxide were gradually added until phenolphthalein paper turned distinctly red. The excess barium hydroxide was removed with carbon dioxide, and the filtrate concentrated to about 30 cc.[5] in the vacuum of a water pump at a bath temperature of 35–40°. The solution was filtered and heated in a boiling water bath for 20 to 25 minutes. The barium salt, precipitated at the boiling point, was sucked off while still hot, and dried over phosphorus pentoxide in a high vacuum. Yield, 3.5 gm. (36.7 per cent of the theoretical).

---

[5] The concentrated solution should not be kept overnight because of the danger of precipitation.

$C_3H_7O_6PBa$ (307.5). Calculated, C 11.7, H 2.3; found, C 11.7, H 2.5

Diethyl Ether of Ethyl l(—)-α-Glycerophosphate



$$\begin{array}{lll} & * & \\ CH_2 & —CH & —CH_2O\cdot PO(OC_2H_5)_2 \\ | & | & \\ O & O & \\ | & | & \\ C_2H_5 & C_2H_5 & \end{array}$$

Silver l(—)-α-Glycerophosphate[6]—A solution of 3.1 gm. of barium salt of acetone l(—)-α-glycerophosphoric acid ($3H_2O$) in 75 cc. of water was treated with 21 cc. of 1 N sulfuric acid, and heated for 15 minutes in a boiling water bath. After cooling to room temperature, 4.2 gm. of crystalline barium hydroxide were added; the excess was removed with carbon dioxide, and the filtrate evaporated to dryness in a vacuum at 30–40°. The residue was dissolved in 25 cc. of distilled water and treated in the dark with a saturated aqueous solution of 3.1 gm. of silver nitrate. The crystalline colorless precipitate of the silver salt was sucked off likewise in the dark, washed with alcohol and ether, and dried in a high vacuum. Yield, 2.24 gm. (75.2 per cent of the theoretical).

$C_3H_7O_6PAg_2$ (385.8). Calculated, Ag 56.3; found, Ag 56.2

Optical Rotation in Dilute Ammoniacal Solution—233.4 mg. of silver salt were suspended in water and just sufficient concentrated ammonia added drop by drop until the solution was almost completely clear. The mixture was made up to 2 cc. and centrifuged. $c = 11.62$, 1 dm. tube, $\alpha_D = +0.12°$, $[\alpha]_D = +1.0°$ ($\pm 0.15°$).

---

[6] Prepared according to the directions of Fischer and Pfähler for the corresponding inactive substance (11). The difficultly soluble barium α-glycerophosphate, which is best prepared according to the simplified method (see above), can also be employed. This salt is dissolved in just sufficient water, and the solution concentrated *in vacuo* at 30–35° and allowed to react with the corresponding amount of silver nitrate solution.

Ethylation—To 2.6 gm. of silver salt[7] were added 4 cc. (7.7 gm.) of ethyl iodide in several portions, a reflux condenser with a ground glass joint being used. After the reaction had subsided somewhat a further 8 cc. of ethyl iodide and 5 gm. of silver oxide were added and the whole refluxed for 4 hours on a water bath. After the mixture had cooled down it was diluted with absolute ether, filtered, and the residue extracted several times with ether. The united ether extracts were dried with sodium sulfate in the presence of a small quantity of silver oxide, and the solvent with the ethyl iodide distilled off through a fractionating column. The residue was twice distilled in a high vacuum. In the second distillation 1.42 gm. (74 per cent of the theoretical) of a clear, colorless, mobile liquid came off. B.p. (0.13 mm.) = 100–100.5° (bath temperature = 110–115°), b.p. (0.03 mm.) = 92–93° (bath temperature 100–105°), $n_D^{19} = 1.4260$.

$C_{11}H_{25}O_6P$ (284.2). Calculated. C 46.4, H 8.8, P 11.00
Found. " 45.4, " 8.7, " 10.92

Optical Rotation—(1) Homogeneous substance in a 1 dm. tube, d_4^{19} = 1.0778, $\alpha_D^{19} = -5.72°$, $[\alpha]_D^{19} = -5.31°$; (2) (*a*) in *absolute* ethyl alcohol (distilled over sodium), 312.2 mg. in 2 cc., $c = 15.61$, 1 dm. tube, $\alpha_D = -0.90°$, $[\alpha]_D^{19} = -5.76°$; (*b*) 307.5 mg. in 2 cc., $c = 15.38$, 1 dm. tube, $\alpha_D = -0.87°$, $[\alpha]_D = -5.66°$; (3) in 92 per cent ethyl alcohol, 289.2 mg. in 2 cc., $c = 14.46$, 1 dm. tube, $\alpha_D = -0.61°$, $[\alpha]_D = -4.22°$. A second preparation gave the following value in absolute ethyl alcohol: 331.7 mg. in 2 cc., $c = 16.58$, 1 dm. tube, $\alpha_D = -0.95°$, $[\alpha]_D = -5.73°$.

The optical rotation of the diethyl ether of diethyl *l*(—)-α-glycerophosphate is strongly dependent on the water content of the alcohol used as solvent.

Dimethyl Ether of Methyl l(—)-α-Glycerophosphate

1.3 gm. of the silver salt[6] of *l*(—)-α-glycerophosphoric acid yielded 0.75 gm. (97 per cent of the theoretical) of the dimethyl ether dimethyl ester, prepared according to the prescription of Karrer and Salomon (5). B. p. (0.13 mm.) = 87–88° (bath 110°), b. p. (0.04 mm.) = 80–81° (bath 100–104°).

$C_7H_{17}O_6P$ (228.16). Calculated, C 36.8, H 7.5; found, C 36.8, H 7.4

Optical Rotation—In absolute ethyl alcohol (distilled over sodium), 0.2930 gm. in 2 cc., $c = 14.65$, 1 dm. tube, $\alpha_D = -0.70°$, $[\alpha]_D = -4.78°$.

---

[7] Prepared from the barium α-glycerophosphate obtained according to the simplified procedure.

For the same product Meyerhof and Kiessling (7) found $[\alpha]_D =$ $-4.46°$ (C_2H_5OH absolute).

SUMMARY

1. With *d*(+)-acetone glycerol as starting material, the *l*(−)-α-glycerophosphoric acid has been prepared, which according to Meyerhof and Kiessling is biologically utilized to the extent of 100 per cent.

2. Its steric relationship to the series of the *l*-glyceraldehyde has been proved, and several biological conclusions have been drawn. The biologically unutilizable *d*(+)-α-glycerophosphoric acid must be related to the *d*-glyceraldehyde.

BIBLIOGRAPHY

1. Fischer, H. O. L., and Baer, E., *Naturwissenschaften*, **25**, 588, 589 (1937).
2. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **128**, 475 (1939).
3. Grün, A., in Schönfeld, H., Chemie und Technologie der Fette und Fettprodukte, Vienna, **1**, 264 (1936).
4. Thierfelder, H., and Klenk, E., Die Chemie der Cerebroside und Phosphatide, Berlin (1930).
5. Karrer, P., and Salomon, H., *Helv. chim. acta*, **9**, 3 (1926).
6. Grimbert, L., and Bailly, O., *Compt. rend. Acad.*, **160**, 207 (1915). Bailly, O., *Compt. rend. Acad.*, **160**, 395, 663 (1915); *Rev. gén. sc.*, **29**, 208 (1918); *Bull. Soc. chim. biol.*, **1**, 152 (1919).
7. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **264**, 46 (1933).
8. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **264**, 62 (1933); **267**, 330 (1933).
9. Abderhalden, E., and Eichwald, E., *Ber. chem. Ges.*, **51**, 1308 (1918).
10. Karrer, P., and Benz, P., *Helv. chim. acta*, **9**, 23 (1926).
11. Fischer, E., and Pfähler, E., *Ber. chem. Ges.*, **53**, 1615 (1920).
12. Malaprade, L., *Bull. Soc. chim.*, **43**, 683 (1928).
13. Kiessling, W., and Schuster, P., *Ber. chem. Ges.*, **71**, 123 (1938).
14. Meyer, H., Analyse und Konstitutionsermittlung organischer Verbindungen, Berlin, 4th edition, 352 (1922).
15. Messinger, J., *Ber. chem. Ges.*, **21**, 3366 (1888).

THE RELATION OF PYRROLE-CONTAINING PIGMENTS TO HEMOGLOBIN SYNTHESIS*

BY G. O. KOHLER, C. A. ELVEHJEM, AND E. B. HART

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, March 4, 1939)

During the past several years the authors have been interested in the nutritive properties of green plant tissues. Experiments carried out during this period have resulted in the discovery of a previously unknown water-soluble growth factor, tentatively named the "grass juice factor" (1). Parallel with these studies, we have attempted to determine the possible nutritional significance of the fat-soluble plant pigment chlorophyll.

The close structural relationship between chlorophyll and the prosthetic group of hemoglobin suggested to Bürgi and von Traczewski (2-4) that the former pigment might be effective in the cure of anemia. Although a great deal of research has been carried out in attempts to prove or disprove this hypothesis, clear cut results have not been forthcoming. Various workers have reported curative effects of chlorophyll or its derivatives in anemias of the following types, protein deficiency (5), hemorrhagic (6, 7), phenylhydrazine poisoning (3, 8), pernicious (9), hypochromic of unknown etiology (9-11), and "experimental nutritional anemia" of undefined character (12). Some of the reports are based on clinical studies, while others are the result of animal experimentation. Erythropoietic effects of chlorophyll in presumably normal animals have also been reported (12, 13). Some of the workers cited have claimed that the chlorophyll was being converted into hemoglobin, while others believe that its beneficial

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by a grant from the Cerophyl Laboratories, Division of the American Dairies, Kansas City.

action was due to its stimulating effect on the hematopoietic centers. Chemical investigations on the biological degradation products of chlorophyll have indicated that conversion to the simpler porphyrin III series does not occur (14, 15). Similar claims of hematopoietic activity have been made for protoporphyrin (16), hemoglobin (17), and bilirubin (18, 19).

A possible explanation of the discrepancies lies in the fact that too little attention has been given to the limiting factor for hemoglobin building in the anemia in question. Thus in simple hemorrhagic anemia, hemoglobin responses can be obtained by the addition of pure iron, pure copper, or by the use of certain liver fractions, depending upon the previous nutritional history of the experimental animal. It is quite possible that chlorophyll might be effective under one set of conditions but not under another. In none of the previous work have the conditions been adequately controlled. Furthermore, no evidence was given in the papers cited above to indicate that the preparations used were free from iron and copper.

It is obvious that hematin precursors could not be built into hemoglobin in the absence of iron, since iron is a part of the hemoglobin molecule. In the case of copper, however, it seemed possible that chlorophyll or other tetrapyrrole pigments might be of value, since one of the functions of copper seems to be associated with the synthesis of the tetrapyrrole nucleus. Thus in copper deficiency in plants, chlorophyll synthesis is decreased (20), in copper-deficient yeast and animals the cytochrome A content of the cells is reduced (21, 22), and in copper-deficient animals, hemoglobin cannot be built.

The present paper describes the results of experiments designed to show whether copper-deficient animals can utilize porphyrin derivatives, *e.g.* chlorophyll, protoporphyrin, and bilirubin, for the synthesis of hemoglobin. Further, a study has been made of the fecal pigments of rats which had been fed chlorophyll.

EXPERIMENTAL

Effect of Tetrapyrrole Compounds on Anemia of Copper Deficiency—The method of Elvehjem and Kemmerer (23) was used to produce severe milk anemia in rats. 3 weeks after weaning, when the hemoglobin levels had fallen to 2 to 4 gm. per 100 cc.

of blood, each rat was fed 0.5 mg. of iron daily. The $FeCl_3$ used was especially purified to free it of copper. Some rats showed no hemoglobin response at all after 1 week of iron therapy. These were ready for pigment feeding. Others showed a slight temporary increase in hemoglobin level, and hence were continued on the milk and iron diet until their hemoglobin values started to fall again, at which time the pigments were tested. Chlorophyll[1] and protoporphyrin[2] were fed at levels of 20 mg. and 40 mg. per day. Iron feeding was continued throughout all of the experiments and hemoglobin determinations were made weekly. The typical data in Fig. 1 show that hemoglobin production did not increase. Since it is known that porphyrin derivatives are poorly absorbed by animals, the compounds were then given to copper-deficient rats by parenteral injection. Chlorophyll dissolved

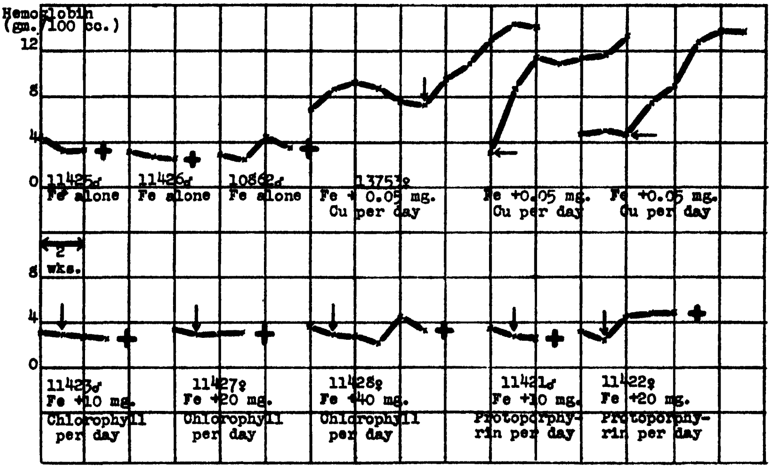


FIG. 1. The effect of copper, chlorophyll, and protoporphyrin, *per os*, on copper deficiency anemia. All animals received 0.5 mg. of iron daily in addition to milk *ad libitum*. The arrows indicate the start of pigment or copper therapy. The cross indicates the death of the animal.

---

[1] The chlorophyll used was grade No. 5X and was obtained from American Chlorophyll, Inc. The ratio of chlorophyll A to chlorophyll B was 3:1.

[2] The protoporphyrin was prepared by Dr. M. O. Schultze, Department of Chemistry, University of Pittsburgh.

in olive oil was injected in amounts ranging from 20 to 75 mg. Control rats received the olive oil alone. Another series of rats was injected with protoporphyrin at levels of 20 and 30 mg. For these injections the sodium salt of protoporphyrin was dissolved in water and the solution neutralized to pH 7.0. Still another group of rats received bilirubin[3] intraperitoneally in amounts of 15 to 50 mg. over a period of several days. For injection the

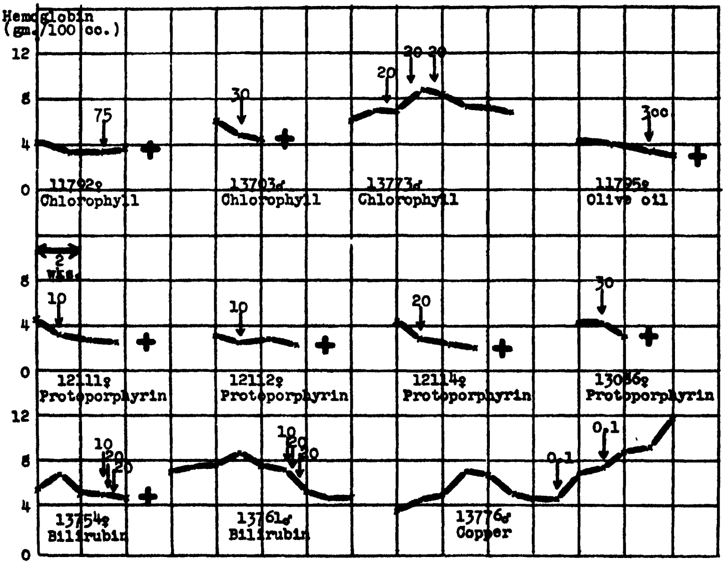


FIG. 2. The effect of parenteral injections of copper, chlorophyll, protoporphyrin, and bilirubin on copper deficiency anemia. All animals received 0.5 mg. of iron daily in addition to milk *ad libitum*. The arrows indicate single injections of the material in question. The amounts injected are expressed in mg. The cross indicates the death of the animal.

bilirubin was suspended in a copper-free phosphate buffer at pH 7.5. The data presented in Fig. 2 show that in none of the cases did porphyrin derivatives have an appreciable effect on hemoglobin building.

Fate of Chlorophyll in Gastrointestinal Tract of Rat—Soon after the beginning of the experiments described above, it was observed that the feces of the rats receiving chlorophyll *per os* were dark green in color. In order to determine the nature of the green pigments, the following procedure was carried out. Two of the rats which were receiving 20 mg. and 40 mg. of chlorophyll per day were placed in metabolism cages. Their feces were collected over a 1 week period, the 2nd week of chlorophyll feeding. These feces were then dried, ground in a mortar, and finally extracted for 24 hours in a Soxhlet extractor in a mixture of 16 parts of skellysolve B, 5 parts of methyl alcohol, and 1 part of benzene. The dark green extract was shaken several times with water to remove the methyl alcohol. The petroleum ether-benzene phase was separated, dried with anhydrous sodium carbonate, and finally made up to a volume of 1 liter with a 16:1 mixture of petroleum ether and benzene. A 20 cc. aliquot of this solution was passed through a chromatogram with anhydrous sodium sulfate as adsorbent. The chromatogram column was prepared as described by Hesse (24). The dark green pigment band which appeared at the top of the column was resolved into four well defined bands and several faint indistinct bands by continued washing with a mixture of petroleum ether and benzene (9:1). The sodium sulfate column was then carefully removed from the glass tube and the layers of pigments separated by sectioning the column with a penknife. The two layers nearest the top, apparently chlorophyll A and chlorophyll B, were completely separated from the other pigments but not separated from each other. The chlorophyll fraction was eluted with acetone and the absorption spectrum of the acetone solution was compared with that of a standard solution containing 3 mg. of chlorophyll per liter (ratio of chlorophyll A to B = 3:1). The absorption curves of the feces pigment and the standard corresponded closely in form. The ratio of absorption intensities at 437.5 mμ was equal within the limit of experimental error, to the ratio at 665.0 mμ, and hence it may be concluded that the pigments of the upper bands of the chromatogram were chlorophylls A and B. Calculations based on the light absorption at these wave-lengths show that 53 per cent of the chlorophyll ingested by the animal receiving 20 mg. per day had passed through the gastrointestinal tract unchanged. For the animal receiving 40 mg. per day the figure was 57 per cent.

---

[3] The bilirubin was purchased from the Eastman Kodak Company, Rochester, New York.

The third layer of the chromatogram, which was apparently not homogeneous, was placed in a mixture of distilled water and petroleum ether. Upon shaking the sodium sulfate dissolved in the water and the pigment was transferred to the petroleum ether phase. After the latter fraction was shaken with successive portions of water and the phases separated in a separatory funnel, the solution of pigment was dried with anhydrous Na_2CO_3 and again passed through a chromatogram, this time with powdered sugar as adsorbent. Washing with 9:1 and finally with 4:1 petroleum ether-benzene mixture allowed the separation from the main pigment of two other pigments, one of which appeared above it in the chromatogram while the other appeared below it. The layers were separated as described above and the main pigment eluted by the use of a mixture of distilled water and ether. The absorption maxima are given in Table I, together with those of the four probophorbides isolated by Fischer and Hendschel (15) from sheep feces. Comparison indicates that the pigment of the third band of the chromatogram was either probophorbide A or C. These two compounds cannot be distinguished spectrophotometrically.

TABLE I

Absorption Maxima in Mμ of Chlorophyll Degradation Products (in Ether)

Absorption Band	I	II	III	IV	V
Third band of chromatogram*	502.5	532.5	564 ± 6.0	605.0	665.0
Fourth band of chromatogram*	497.5	535.0	560.0	608.8 ± 5.0	665.0
Probophorbide A (15)	503.3	535.3	558.0	605.0	665.6
" B (15)	498.3	532.1	561.9	609.4	665.3
" C (15)	504.2	535.0	561.3	605.7	664.6
" D (15)	500.7	534.4	561.9	600.0	663.4

The numbers in parentheses refer to the bibliography.

* The maximum error unless otherwise indicated is ±2.5 mμ.

The pigment of the fourth band of the chromatogram was purified by being passed through a chromatogram in a similar manner. Its absorption spectrum, the maxima of which are also included in Table I, corresponds closely to that of probophorbide B.

The faint bands of the original chromatogram were not investigated.

DISCUSSION

The work of Barkan (25) and of Lemberg and coworkers (26) has shown that in the breakdown of hemoglobin to bilirubin, two intermediates, α- and β-pseudohemoglobin, are formed. The discovery by Edlbacher and von Segesser (27) of a copper-activated enzyme in hemolyzed red blood cells which catalyzed the production of pseudohemoglobins from hemoglobin immediately suggested that in the intact red blood cell, the reaction might proceed in the opposite direction. The results of the experiments reported in this paper would be expected if this theory were true, since then copper would be necessary for the last step in the process of hematopoiesis; namely, for the conversion of pseudohemoglobin to hemoglobin. Although it seems possible that this is one of the functions of copper, it is rather unlikely that this is the only function, especially in view of the fact that we now recognize definite enzymes such as tyrosinase and polyphenol oxidase which contain copper (28, 29). A disturbance in the oxidation-reduction systems of the hematopoietic centers might well result in decreased hemoglobin production.

The degradation of chlorophyll in the gastrointestinal tracts of various species, especially of Herbivora, has been the subject of much research. The probophorbides have been found in human feces (15), sheep feces (15), sheep stomach contents (30), elephant feces (15), and horse intestinal concretions (31). The work reported in the present paper indicates that degradation of chlorophyll in the rat intestine resembles that which occurs in human intestines (15, 32) in that in both cases the degradation proceeds to a much lesser extent than it does in ruminants, in which the end-product is phylloerythrin.

The work of Fischer and Stadler (33) has shown that natural probophorbide A is a mixture of pyropheophorbide A and dihydropyropheophorbide A, while probophorbide B is chiefly pyropheophorbide B. Thus it appears that in the rat intestine, degradation of chlorophyll for the most part consists in removal of magnesium, phytyl alcohol, and methyl alcohol, and decarboxylation at C_{10}. Hydrogenation of the vinyl group occurs to some extent.

SUMMARY

1. Anemic rats deficient in copper but receiving optimum quantities of iron were given chlorophyll, protoporphyrin, and bilirubin, both *per os* and parenterally. None of these compounds had a beneficial effect on hemoglobin production.
2. The feces pigments of rats receiving chlorophyll *per os* were separated by means of the chromatogram.
3. At the level fed, between 50 and 60 per cent of the chlorophyll ingested was excreted unchanged in the feces.
4. The chlorophyll not eliminated *per se* appeared in the feces chiefly in the form of probophorbide A (or C) and probophorbide B.
5. The relation of copper to the synthesis of hemoglobin in the light of recent discoveries is briefly discussed.

BIBLIOGRAPHY

1. Kohler, G. O., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **14**, 131 (1937); **15**, 445 (1938).
2. Bürgi, E., *Korr.-Bl. schweitz. Ärtze*, **46**, 449 (1916).
3. Bürgi, E., and von Traczewski, C. F., *Biochem. Z.*, **98**, 256 (1919).
4. Bürgi, E., Das Chlorophyll als Pharmakon, Leipzig (1932).
5. Saunders, C. W., *Proc. Soc. Exp. Biol. and Med.*, **23**, 788 (1925).
6. Binet, L., and Strumza, M. V., *Sang*, **8**, 1041 (1934).
7. Hughes, J. H., and Latner, A. L., *J. Physiol.*, **86**, 388 (1936).
8. Aoki, S., *Sei-i-Kwai Med. J.*, **50**, 1 (1931); *Chem. Abst.*, **26**, 3847 (1932).
9. Zichgraf, G., *Münch. med. Woch.*, **79**, 998 (1932).
10. Schweitzer, A., *Wein. med. Woch.*, **85**, 727 (1935).
11. Patek, A. J., *Arch. Int. Med.*, **57**, 73 (1936).
12. Rothemund, P., McNary, R. R., and Inman, O. L., *J. Am. Chem. Soc.*, **56**, 2400 (1934).
13. Zih, A., *Arch. ges. Physiol.*, **231**, 510 (1933).
14. Marchlewski, L., and Urbańczyk, W., *Biochem. Z.*, **263**, 166 (1933).
15. Fischer, H., and Hendschel, A., *Z. physiol. Chem.*, **198**, 33 (1931); **206**, 255 (1932); **216**, 57 (1933); **222**, 250 (1933).
16. Hinsberg, K., and Merten, R., *Biochem. Z.*, **299**, 137 (1938).
17. Zih, A., *Arch. ges. Physiol.*, **231**, 514 (1933).
18. Bencsik, F., Gáspár, A., Verzár, F., and Zih, A., *Biochem. Z.*, **225**, 278 (1930).
19. Patek, A. J., and Minot, G. R., *Am. J. Med. Sc.*, **188**, 206 (1934).
20. Orth, O. S., Wickwire, G. C., and Burge, W. E., *Science*, **79**, 33 (1934).
21. Elvehjem, C. A., *J. Biol. Chem.*, **90**, 111 (1931).
22. Cohen, E., and Elvehjem, C. A., *J. Biol. Chem.*, **107**, 97 (1934).
23. Elvehjem, C. A., and Kemmerer, A. R., *J. Biol. Chem.*, **93**, 189 (1931).
24. Hesse, G., *Angew. Chem.*, **49**, 315 (1936).

25. Barkan, G., *Klin. Woch.*, **16**, 1265 (1937).
26. Lemberg, R., Legge, J. W., and Lockwood, W. H., *Nature*, **142**, 148 (1938).
27. Edlbacher, S., and von Segesser, A., *Naturwissenschaften*, **34**, 557 (1937).
28. Dalton, H. R., and Nelson, J. M., *J. Am. Chem. Soc.*, **60**, 3085 (1938).
29. Kubowitz, F., *Biochem. Z.*, **292**, 221 (1937); **296**, 443 (1938).
30. Rothemund, P., and Inman, O. L., *J. Am. Chem. Soc.*, **54**, 4702 (1932).
31. Triebs, A., *Z. physiol. Chem.*, **220**, 89 (1933).
32. Brugsch, J. T., and Sheard, C., *J. Lab. and Clin. Med.*, **24**, 230 (1938).
33. Fischer, H., and Stadler, F., *Z. physiol. Chem.*, **239**, 167 (1936).

SPECTROPHOTOMETRIC EXAMINATION OF BLOOD FROM ANIMALS RECEIVING SULFANILAMIDE

By T. J. WEBB and MICHAEL KNIAZUK

(*From the Research Laboratories of Merck and Company, Inc., and the Merck Institute of Therapeutic Research, Rahway*)

(Received for publication, January 31, 1939)

A series of experiments was undertaken with the purpose of determining, if possible, what foreign pigments are present in the blood of animals exhibiting the marked cyanosis which frequently accompanies the administration of sulfanilamide. The presence of sulfhemoglobin or methemoglobin (or both) has been reported by many observers. Among such reports, however, there has been little unanimity, and moreover other observers have reported cases of clinical cyanosis unaccompanied by detectable quantities of non-functional iron pigment (1). The problem appeared to be one on which considerable light might be thrown by a comprehensive spectrophotometric investigation. In view of the confusion existing in this field, it was felt from the start that little could be accomplished towards clarification unless complete absorption curves for the visible range were obtained. Moreover, the highest attainable accuracy seemed to be required.

Technique of Measurement and Standards for Normal Blood

A total of 81 rats was used in this investigation. In all, 112 samples of blood were examined. Sulfanilamide in the form of a 10 per cent suspension (with 5 per cent gum acacia) was administered by stomach tube. The daily dose was 2 gm. per kilo.[1] 0.5 cc. of blood was withdrawn by means of a syringe from the heart of the unfed animal, delivered into 0.25 cc. of a 0.5 per cent sodium oxalate solution, and centrifuged. The supernatant liquid was removed and the erythrocytes washed three times with physiological salt solution, each washing being followed by centrifuging and removal of the salt solution. The washed erythrocytes were hemolyzed with distilled water and the total volume brought to 50 cc. The solution was then centrifuged at high speed for 2 hours in order to clear the solution for photometric reading. These solutions were diluted 3-fold further with buffers of the desired pH. The readings were made with a Bausch and Lomb spectrophotometer, 10 cm. tubes being used for the 6000 to 6600 Å. spectral region and 2 cm. tubes for the 5200 to 6000 Å. region.[2] All measurements were made within 6 hours after the blood was drawn. The spectrophotometric readings were reproducible to within 2 per cent, and the over-all reproducibility of the measurements was estimated to be well within 5 per cent.

---

[1] When there seemed to be danger of the animal dying, the dose was reduced to 1.5 gm. per kilo.

TABLE I

Extinction Coefficient

Å.	Average	High	Low	$\frac{\alpha}{\varphi}$	$\frac{\beta}{\varphi}$
5770	15.13	15.26	15.06	1.73	
5600	8.73	8.95	8.56		
5420	14.62	14.70	14.52		1.68

For purposes of standardization, a sample of each of the blood solutions was treated with $K_3Fe(CN)_6$ and KCN according to the method of Drabkin and Austin (2) and the extinction measured at 5450 Å. By this procedure, oxyhemoglobin as well as any methemoglobin which may be present is converted to cyanmethemoglobin. The millimolecular extinction coefficient ϵ_m[*] is 11.5 at 5450 Å. for this compound. The measurement of the extinction at this wave-length thus provides a means of determining accurately the hemoglobin content of blood solutions (not contaminated with pigments other than methemoglobin) and hence of computing the extinction coefficient, ϵ_m (c = 1 mM per liter). This method of computation was followed in all cases. In cases of colored contaminants (other than methemoglobin) this method of computation is necessarily arbitrary. In such cases the relative extinctions so computed are denoted by ϵ'_m.

---

[2] In some instances, readings were made with a photoelectric indicator devised by one of us (M. Kniazuk). Details will appear in a forthcoming article.

[*] $-\mathrm{Log}_{10} T = \epsilon_m c l$, in which ϵ_m is the millimolecular extinction coefficient, c is the concentration in mM (16.7 gm. in the case of hemoglobin) per liter, and l is the length in cm.

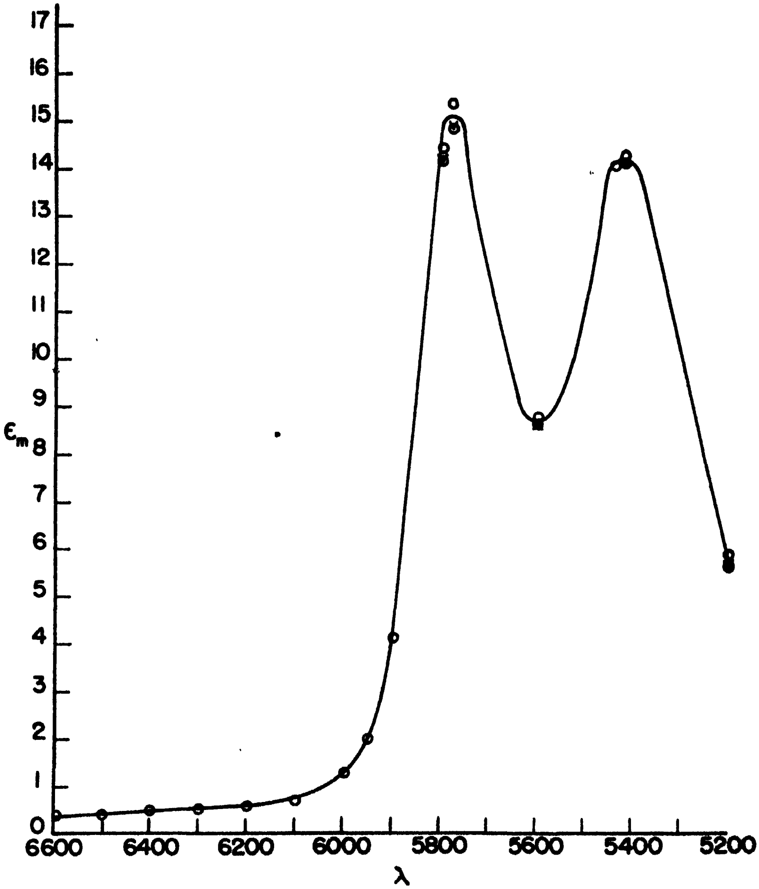


Fig. 1. Absorption curve for oxyhemoglobin. Circles indicate measurements at pH 9; circles with arrow-heads, measurements at pH 7. When measurements under the different conditions agree exactly, only plain circles appear.

The precision measurements of Drabkin and Austin (2) were considered standard criteria for spectrophotometric determinations of hemoglobin and related compounds.[3] These authors report the values recorded in Table I for the absorption characteristics of oxyhemoglobin in neutral aqueous solution at various wavelengths. α/φ, β/φ are respectively the ratios of the extinctions at 5770 and 5420 Å. to that at 5600 Å. No dependence of the extinction on the pH is indicated in their work.

A typical absorption curve for an oxyhemoglobin solution, obtained from washed erythrocytes according to the technique described above, is given in Fig. 1. It is to be noted that in the spectral region 6600 to 6000 Å. the measurements at pH 7 and 9 agree exactly, and that in the region of the absorption bands of sulf- and methemoglobin (6200 to 6400 Å.), the extinction coefficient is 0.5 ± 0.1. It is to be noted further that in the spectral region 5400 to 5800 Å. there is a slight dependence on the pH. In spite of this observed dependence on the pH (within the experimental error) the values of the extinction fall inside the limits set by Drabkin and Austin. Complete independence of the pH was regarded as normal for oxyhemoglobin solutions, although no sample was regarded as significantly abnormal in this respect unless the difference in absorption, for pH 7 and 9, amounted to as much as 0.2 in the 6100 to 6400 Å. range and to as much as 0.5 at the wave-lengths 5770, 5600, and 5420 Å. This latter latitude had to be allowed, since with normal bloods differences as great as 0.5 were frequently observed for solutions at pH 7 and 9, the solution at pH 7 invariably giving rise to a smaller absorption than that at pH 9 (in the 5400 to 5800 Å. region) when they did not coincide. This effect was investigated at length and regarded finally as a normal variation. The α/φ ratio was regarded as normal if it lay between 1.60 and 1.75 and the β/φ ratio as normal if it lay approximately 0.05 below the α/φ value. Addition of KCN was observed not to alter the absorption of oxyhemoglobin solutions. This test was applied regularly in the 6000 to 6600 Å. region, where alterations in the absorption would be most indicative.

---

[3] Reports of slight differences in the absorption curves of hemoglobin of different species have not been considered significant for the present purpose.

Results Obtained Following Sulfanilamide Administration

A large number of bloods indicated the presence of methemoglobin. A typical example is given in Fig. 2. The animal whose blood is represented here had been fed sulfanilamide for only 3 days, but similar curves were obtained in cases of much longer feeding. The presence of methemoglobin seems to be

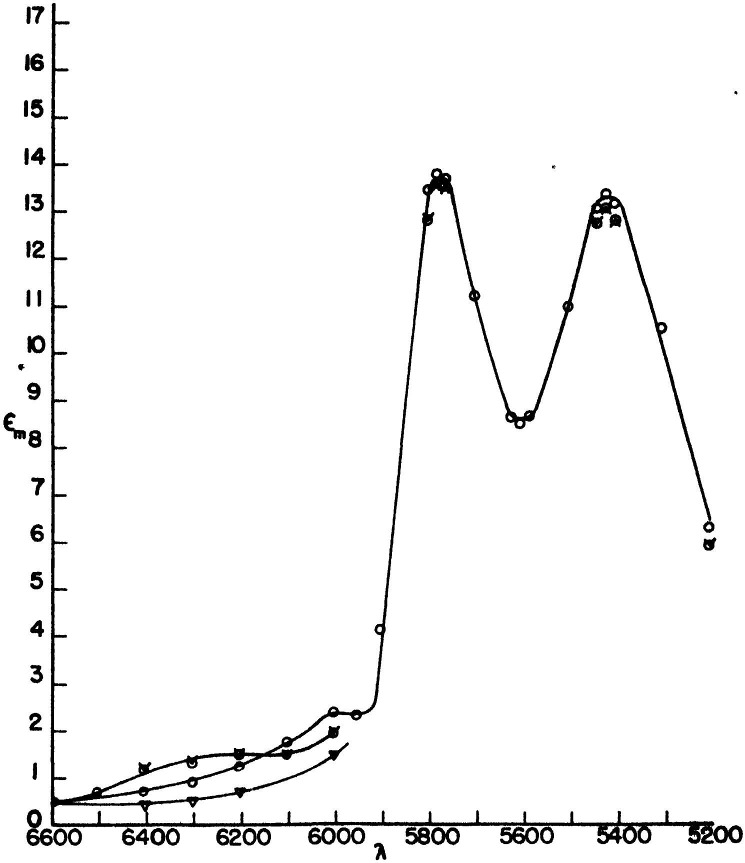


FIG. 2. Absorption curve indicating presence of methemoglobin. Circles indicate measurements at pH 9; circles with arrow-heads, measurements at pH 7; triangles, measurements at pH 9 after treatment with KCN. When measurements under the different conditions agree exactly, only plain circles appear.

definitely indicated by the graph in Fig. 2 for the following reasons: (1) the increase in absorption in the region of the methemoglobin band, 6100 to 6400 Å., (2) the nature of the dependence of the absorption on the pH in this region, (3) the crossing of the absorption curves (for various pH) at 6150 Å., (4) the reduction of the absorption in the 6100 to 6400 Å. region following the addition

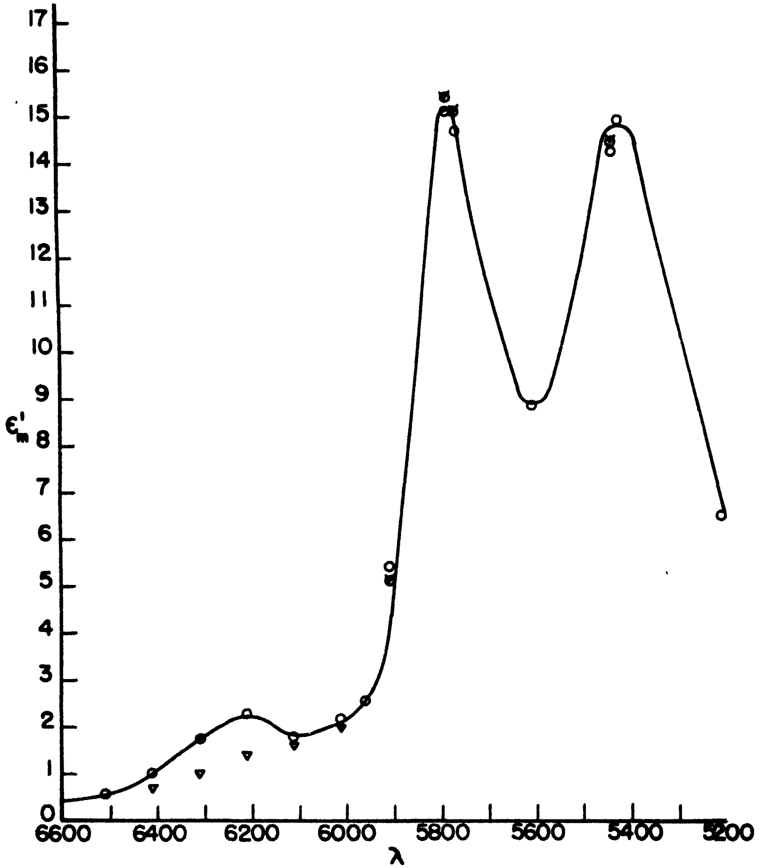


FIG. 3. Absorption curve indicating presence of sulfhemoglobin. Circles indicate measurements at pH 9; circles with arrow-heads, measurements at pH 7; triangles, measurements at pH 9 after treatment with KCN. When measurements under the different conditions agree exactly, only plain circles appear.

of KCN. Graduated production of methemoglobin by adding $K_3Fe(CN)_6$ to oxyhemoglobin solutions yielded absorptions, confirming the facts on which the above conclusions are based.

A large number of bloods gave rise to curves like that given in Fig. 3. The pronounced maximum of absorption at 6200 Å. and the fact that the absorption curves show no dependence on the pH suggest strongly that the contaminating pigment in these cases is sulfhemoglobin. The animal whose blood is represented in Fig. 3 had been fed sulfanilamide for 11 days, but the development of a blood of these characteristics bore no detectable relationship to the duration of feeding. Drabkin and Austin (2) have determined by indirect means an absorption curve for pure sulfhemoglobin. Their curve shows a pronounced maximum at 6200 Å., ϵ_m being equal to 6.0, 11.0, and 7.6 at 6400, 6200, and 6000 Å. respectively. A mixture of 14 per cent sulfhemoglobin and 86 per cent oxyhemoglobin for example shows clearly a maximum of absorption at 6200 Å. ($\epsilon_m = 2.0$; compare $\epsilon_m = 0.5$ for pure oxyhemoglobin). From the height of the maximum at 6200 Å. in Fig. 3, the sulfhemoglobin content is estimated to be approximately 15 per cent.

In Fig. 4 is given a curve showing a similarity to that of Fig. 3; *i.e.*, especially in showing a well defined maximum at the wavelength 6200 Å., which is characteristic of sulfhemoglobin. This curve, however, differs from that in Fig. 3 by showing a dependence on the pH. This dependence on the pH cannot be attributed to methemoglobin, since the curves do not cross at any point in the 6000 to 6600 Å. range, as expected, and moreover regularly observed, in the case of methemoglobin. The scarcely perceptible decrease in the absorption (pH 9) on addition of KCN likewise indicates that the additional contaminant is not methemoglobin. It was assumed, however, that curves of this type indicated that one of the contaminants of the oxyhemoglobin is sulfhemoglobin.

Many strikingly abnormal curves were obtained as shown in Fig. 5. The animals whose bloods are represented here were fed sulfanilamide for 9 days. The abnormalities in these curves are such that they cannot be attributed primarily either to methemoglobin or to sulfhemoglobin. It is to be noted that these curves show a greater absorption at 5420 Å. than at 5770 Å. and a large dependence on the pH over the entire range. Both of these effects are much greater than that which could be produced by methemoglobin (or by sulfhemoglobin), at least in the con-

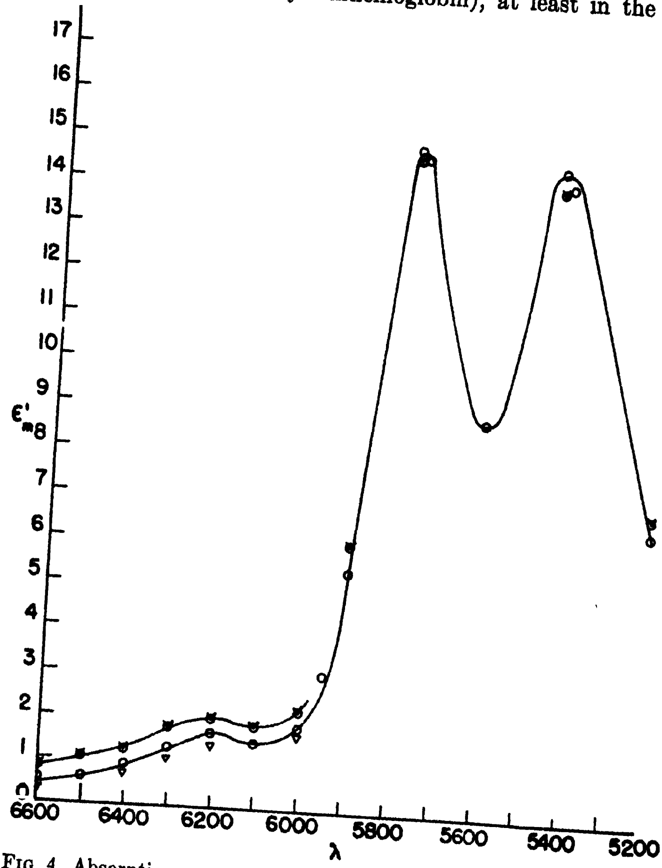


FIG. 4. Absorption curve assumed to indicate presence of sulfhemoglobin plus unidentified pigment. Circles indicate measurements at pH 9; circles with arrow-heads, measurements at pH 7; triangles, measurements at pH 9 after treatment with KCN. When measurements under the different conditions agree exactly, only plain circles appear.

centrations indicated by the absorption observed in the 6100 to 6300 Å. region.[4]

---

[4] In curves departing so widely from that of oxyhemoglobin, it is essential to remember that the ordinates (ϵ'_m) measure relative absorptions only.

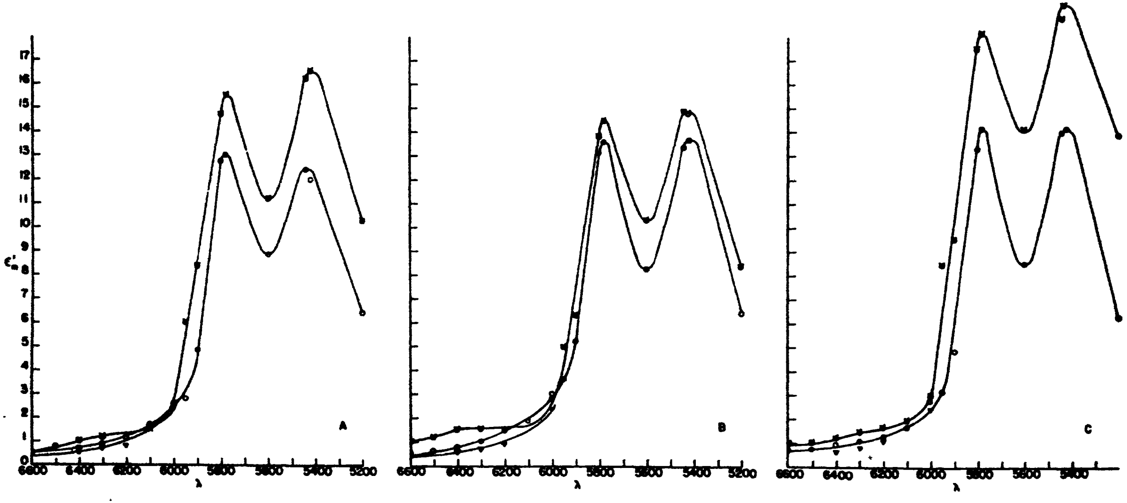


FIG. 5. Absorption curves indicating presence of unidentified pigments. Circles indicate measurements at pH 9; circles with arrow-heads, measurements at pH 7; triangles, measurements at pH 9 after treatment with KCN. When measurements under the different conditions agree exactly, only plain circles appear.

Curves 1 to 9 in Fig. 6 represent on an enlarged scale the spectrophotometric results obtained in the 6100 to 6500 Å. region for

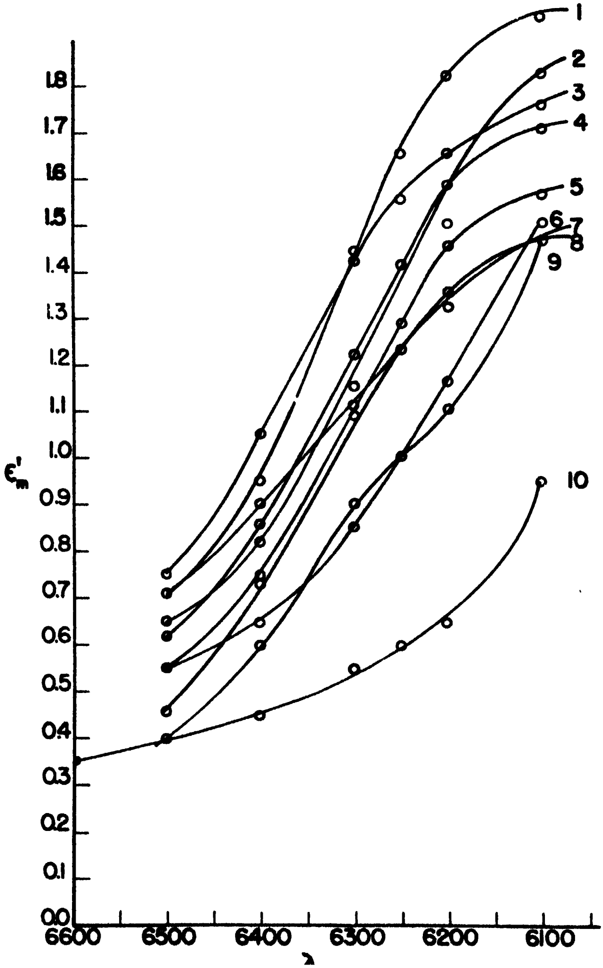


Fig. 6. Absorption curves for the 6100 to 6500 Å. spectral region on an enlarged scale. Curves 1 to 9 represent results obtained for the blood solutions (pH 9) of a group of rats fed sulfanilamide for 62, 43, 30, 27, 62, 58, 18, 21, and 58 days respectively. Curve 10 represents a normal blood.

the blood solutions (pH 9) of a group of rats fed sulfanilamide for 62, 43, 30, 27, 62, 58, 18, 21, and 58 days respectively. Curve

10 represents a normal blood. In all of these solutions the absorption showed a pH dependence, the absorption at pH 7 being appreciably greater than that at pH 9 (in the 6100 to 6500 Å.

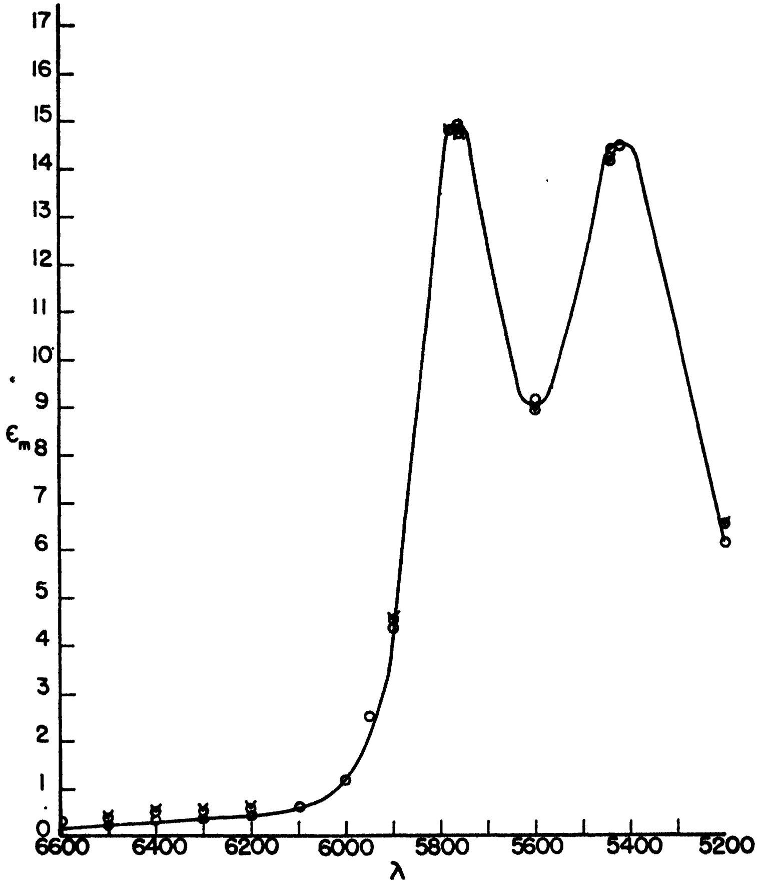


Fig. 7. Absorption curve showing inappreciable departure from that of oxyhemoglobin. Circles indicate measurements at pH 9; circles with arrow-heads, measurements at pH 7. When measurements under the different conditions agree exactly, only plain circles appear.

region), and there was almost invariably a slight reduction in the absorption on addition of KCN. Small percentages of methemoglobin were assumed in all of these solutions, but the general shape of the curves seems to preclude the possibility that the chief contaminant in these solutions was either methemoglobin or sulfhemoglobin (or a mixture of the two).

It should be mentioned finally that many samples of blood were examined which showed scarcely detectable departures from the normal. An example is given in Fig. 7. This apparent absence of foreign pigment was observed repeatedly, even in cases in which the animals showed pronounced cyanosis and in which the blood immediately on being drawn was definitely of a darker, bluish cast than that from normal animals.

DISCUSSION

From the above spectrophotometric results, as well as those reported by other investigators, it is clear that methemoglobin or sulfhemoglobin (or both) may be frequently observed in blood from animals receiving sulfanilamide. It is undeniable, however, that cyanosis occurs in cases in which methemoglobin or sulfhemoglobin (or both) is not present in the blood in quantities large enough for practical detection. Moreover, many blood solutions have been examined which appear to be perfectly normal spectrophotometrically. In others there are present to a comparatively large degree foreign pigments as yet unidentified.

In spite of the fact that all animals were kept under identical conditions, there was a variability in the results of the experiments which seemed to be utterly beyond control. It should be mentioned, however, that with *any* given group of animals being treated simultaneously, the results seemed to be to a certain extent of the same general character, although there was still a wide variation in the degree of the observed abnormality between different members of a group. Moreover, with a given member of the group, the daily variation in the photometric blood picture was distinctly irregular. There was often a considerable decrease in hemoglobin content after feeding the drug for several days, followed by irregular increases. The results seem to indicate that the foreign pigments do not arise from direct chemical action of the drug (or of its decomposition products) with compounds present in the animal body but rather that the drug indirectly stimulates the hematopoietic mechanism, resulting in a chaotic production of compounds related to hemoglobin. This conclusion is in line with the results of Rimington and Hemmings (3) who report abnormally high values for the ether-soluble porphyrin content in the urine of hospital patients treated with sulfanilamide.

SUMMARY

A spectrophotometric examination of blood from animals receiving massive daily doses of sulfanilamide indicated a variety of effects: (1) bloods containing either methemoglobin or sulfhemoglobin, (2) normal bloods, and (3) bloods contaminated with unidentified foreign pigment. A rigorous technique was used in preparing the blood solutions. In spite of this and of an attempt at the highest attainable accuracy in the spectrophotometric readings, there was an extremely wide variability in the results obtained.

BIBLIOGRAPHY

1. Marshall, E. K., Jr., and Walzl, E. M., *Bull. Johns Hopkins Hosp.*, **61**, 140 (1937).
2. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **98**, 719 (1932); **112**, 51, 89, 105 (1935-36). Austin, J. H., and Drabkin, D. L., *J. Biol. Chem.*, **112**, 67 (1935-36).
3. Rimington, C., and Hemmings, A. W., *Lancet*, **1**, 770 (1938).

THE GONADOTROPIC HORMONE OF URINE OF PREGNANCY

I. A SIMPLE METHOD OF EXTRACTION AND PURIFICATION*

BY SAMUEL GURIN, CARL BACHMAN, AND D. WRIGHT WILSON

(From the Departments of Physiological Chemistry and Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, February 24, 1939)

Progress in the study of the gonadotropic hormone of pregnancy urine has long been delayed by lack of dependable methods for its purification.[1] Katzman and Doisy (1) as well as Zondek, Scheibler, and Krabbe (2) have succeeded in preparing gonadotropic products containing more than 1000 rat units per mg., but by processes which could not be standardized and in which the yields were apparently insufficient to make further studies practicable. Meyer (3) has described preparations containing 500 or more rabbit ovulation-producing doses per mg., and appears to be the only investigator who has examined in some detail the chemistry of hormone preparations of this order of activity.

When Browne and Venning in 1936 (4) and Evans, Kohls, and Wonder in 1937 (5) reported that the urinary excretion of gonadotropin in the human is many times greater between the 60th and 80th days than at other periods of pregnancy, it seemed reasonable that urine collected at such a time would be a rich and readily available material for study. With the use of such urine we have developed a simple, inexpensive, and well standardized method for obtaining highly active preparations of gonadotropin in good yield.

---

* Aided by grants from the Penrose Fund of the American Philosophical Society, from the National Research Council, and from the Gynecean Hospital Institute for Gynecological Research, University of Pennsylvania.

[1] The literature up to 1932 is well summarized by Katzman and Doisy (1).

EXPERIMENTAL

Assay Technique—The isolation procedures were checked at each step by quantitative assay of the gonadotropic activity of the various products. For this purpose, carefully selected and isolated mature rabbit does were used, 5 to 25 days after they had cast healthy full term litters. The substance to be assayed was administered in a freshly prepared solution by a single injection into a marginal ear vein. Results were read by inspection of the ovaries 24 to 48 hours later, the presence of freshly ovulated follicles constituting the sole criterion of a "positive" reaction. The unit of activity selected was the minimal effective dose (m.e.d.) proposed by Friedman (6)—*i.e.*, the dose per kilo of body weight of rabbit necessary to cause ovulation.[2]

When proper precautions were observed in the selection of assay animals, our own experience has confirmed Friedman's claims for the uniform sensitivity of the healthy postpartum rabbit. In most of the quantitative assays here recorded, single animals were used at the varying dose levels in determining the range of activity of the gonadotropic fractions tested. In certain critical experiments a larger number of animals was employed, but without alteration of the conclusions arrived at with single animals. Details of our experience with quantitative assay work in the rabbit will be reported elsewhere.

The comparative size of the minimal effective dose in the postpartum rabbit in relation to the more commonly used rat unit of the literature has been determined for pregnancy urine preparations by Rowe, Simond, and Nelson (7), who found that 1 minimal effective dose was approximately equivalent to 1 rat unit.

---

[2] Friedman has shown that the sensitivity of the healthy postpartum rabbit to administered gonadotropin is uniform, per unit of body weight, for all animals weighing more than 2 and less than 4.5 kilos. Provided that his precautions concerning the selection of assay animals are observed, a minimal effective dose per kilo will produce ovulation in more than 90 per cent of the rabbits tested. A dose that is 30 per cent lower than this will produce ovulation in less than 10 per cent of animals. Because of this, it is not feasible to select for the rabbit, as is done for the rat, a unit defined by a 50 or 60 per cent incidence of positive reactions. From his statistical data Friedman calculates that with the use of one rabbit at each dose level in any range-finding assay it is possible to define biologic activity nine times out of ten with an error of less than 30 per cent.

A ratio of 1 minimal effective dose to between 1 and 2 rat-luteinizing units has also been suggested by studies which were conducted through an exchange of materials and assay data among Dr. W. O. Nelson, Dr. O. Kamm, Dr. M. H. Friedman, and ourselves.[3]

Source Material and Initial Work-Up—Pregnancy urines were collected between the 50th and 90th days following the last menstrual period.[4] They were commonly kept 1 or 2 days at 0–5° with chloroform, and then each patient's urine was pooled and worked up separately.

For the initial separation of hormone, Katzman and Doisy's benzoic acid adsorption method (8) was employed, all steps being carried out in the cold room at 0–5°. The initial precipitate was washed three or four times with cold, saturated aqueous benzoic acid solution on a Buchner funnel, and the thoroughly pressed precipitate then treated with sufficient acetone to dissolve the benzoic acid. The insoluble residue was washed and dehydrated with several changes of anhydrous acetone, and the product collected and dried *in vacuo*. This material was called Product A.

The following comments on this technique may be of interest: Prolonged washing of the initial precipitate with saturated aqueous benzoic acid solution does not dissolve the hormone, but causes peptization of the hormone-containing residue in the acetone which is used for removing benzoic acid. Unless care is exercised to flocculate this suspended material by adding salt to the acetone, there may be difficulty in collecting it by either filtration or centrifugation, and an appreciable part of the original hormonal activity of the urine may thus be lost. As much as 75 per cent of the available activity of some urines has been recovered from the cloudy supernatant acetone, when the suspended material has been flocculated and collected separately. Such material may show an increased activity amounting to 100 to 250 minimal effective doses per mg. (see Table IV, Experiments 2 and 3). There appears to be no advantage, however, in regularly collecting it apart from the heavier portions of the insoluble material, since the amounts and activities thus recovered are variable.

---

[3] Our thanks are due Dr. W. O. Nelson of Wayne University, Dr. O. Kamm of Parke, Davis and Company, and Dr. M. H. Friedman of the United States Department of Agriculture, for these exchanges of materials and for the assay work mentioned. We are also indebted to Dr. Friedman for valuable advice and assistance in connection with the rabbit assays.

[4] We thank Dr. F. L. Payne for his cooperation in obtaining early pregnancy urine.

When the insoluble residues were dried in the usual manner, approximately 0.5 gm. (±0.2 gm.) of grayish brown powder was obtained from each liter of urine (Table I). The pooled urines assayed directly contained 10,000 to 50,000 minimal effective doses per liter. The recoveries of activity in the powders have usually been quantitative, as Katzman and Doisy found with mixed pregnancy urine. These preparations, containing 25 to 50 minimal effective doses per mg., were the starting material for our purification experiments.

TABLE I

Efficiency of Benzoic Acid Adsorption and Recoveries of Crude Hormone

Experiment No.	Urine		Benzoic acid adsorbate (Product A)			
	Total volume	Activity	Weight	Purity	Activity recovered	
	liters	*m.e.d.*×1000 per l.*	*mg. per l.*	*m.e.d. per mg.*	*m.e.d. × 1000 per l.*	*per cent*
1	10.0	20	495	40	20	100
2	4.7	20	508	40	20	100
3	4.7	20	563	33	20	100
4	8.8		500	50	25	
5	35.7		540	50	25	
6	13.9		640	50	30	
7	14.8		700	55	40	
8	16.0		470	33	15	

*M.e.d. = minimal effective doses (See "Assay technique").

Fractionation of Product A with Aqueous Acetone—Purification of Product A was first attempted by fractional precipitation with acetone. For this purpose a sample was stirred for several hours with 10 volumes of cold water and allowed to stand for 24 hours. After centrifuging and extracting the residue three times with 3 to 4 volumes of water, most of the activity was found in the turbid supernatant, although 80 per cent of the original solids remained insoluble. Addition of cold acetone to a concentration of 30 per cent precipitated the suspended material, and to the clear supernatant the further addition of acetone to 70 per cent yielded a product containing 250 minimal effective doses per mg. and more than 95 per cent of the original activity.

In order to simplify this process another portion of the same Product A was eluted directly by suspending it in 3 to 4 volumes of cold 30 per cent acetone at 0–5° for 24 hours. After centrifugation and repeated washing of the residue with cold solvent, the combined clear extract (10 volumes) was treated with cold acetone to make a concentration of 70 per cent. The resulting precipitate obtained by centrifugation contained 1000 minimal effective doses per mg. and more than 80 per cent of the original activity. No further increase of purity was obtained by a repetition of the procedure. A preliminary report on this method was made in 1938 (9).

TABLE II

Elution of Benzoic Acid Adsorbates with Aqueous Acetone

Experiment No.	Starting material (Product A)			Final product (Product C)			Original activity recovered		
	Weight	Purity	Total activity	Weight	Purity	Total activity	In Product C	In residue from Product A	Total
	gm.	*m.e.d. per mg.*	*m.e.d. × 1000*	*mg.*	*m.e.d. × 1000 per mg.*	*m.e.d. × 1000*	*per cent*	*per cent*	*per cent*
1	4.43*	45	190	170	1.0	170	90		90
2	8.70*	50	435	293	0.5	150	35	25	60
3	1.34†	50	67	36	0.5	18	25	10	35
4	1.45*	40	58	34	1.5	51	90	15	105
5	10.20‡	40	408	322	0.5	161	40	25	65
6	3.89‡	40	156	63	0.5	32	20	40	60
7	6.29*	33	208	53	1.0	53	25	50	75
8	5.10*	33	168	45	2.0	90	55	40	95
9	5.00*	50	250	92	2.0	184	75	25	100
10	5.26*	50	263	85	2.0	170	65	35	100
11	0.91*	50	46	38	1.0	38	85		85

* 30 per cent acetone; 24 hours extraction.

† 40 per cent acetone; 2 hours extraction.

‡ 30 per cent acetone; 2 hours extraction.

Since a 20-fold purification was obtained by this simple process, we began to apply it routinely to our A products (see Table II). It was soon observed, however, that the method required improvement, since the potency of the resulting preparations varied from 500 to 2000 minimal effective doses per mg. and, in addition, considerable variation in total recoveries of the hormone was noted. No further improvement could be obtained by increasing the concentration of acetone in the solvent or by decreasing the time of extraction.

A number of experiments of this type are summarized in Table II. The results of biological assays upon all the fractions thus obtained revealed the following. When end-products of high potency were obtained, the recoveries of activity were satisfactory. When preparations of a low order of activity were obtained, a considerable percentage of the original activity could not be accounted for when both the derived products and residues were assayed. Since there was no synergistic effect observed when certain of these products and residues were mixed together in appropriate proportions and reassayed, the observed losses of activity had to be ascribed to partial inactivation occurring during the purification procedure.

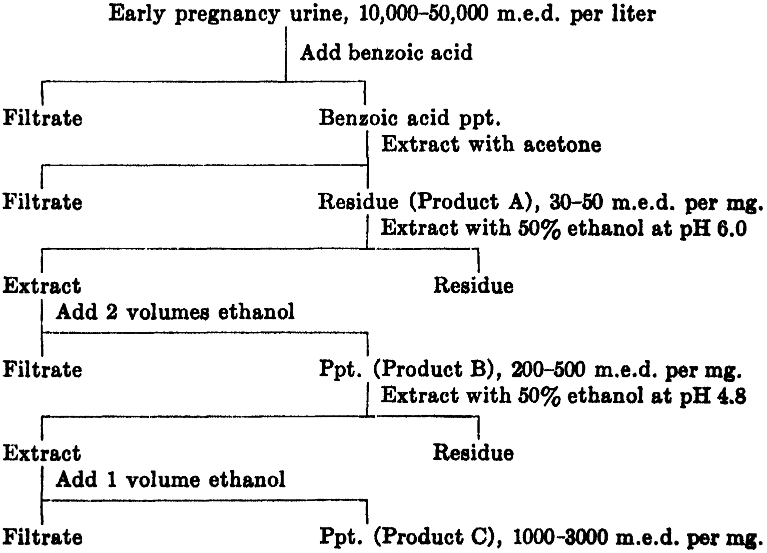
TABLE III

Inactivation of Hormone Solutions at 0-5°

Preparation No.	Solvent	pH*	Activity of solution, m.e.d. per ml.			
			0 hrs.	24 hrs.	48 hrs.	96 hrs.
160	Distilled water	6.5	2.0	2.0		
	Aqueous acetone (30%)	6.5	2.0	2.0		
	Same with CH_3COOH	4.6	2.0	0.5		
	" " C_6H_5COOH	4.6	2.0	0.5		
214C	Distilled water	6.5	2.0		0.5	0.5
	CH_3COOH	3.5	2.0		0.5	0.5
2B	"	4.5	1.0		0.5	0.5
1B	"	4.5	1.0		1.0	0.5

* The pH determinations were made colorimetrically.

Observations on Inactivation of Gonadotropin—Several investigators have noted that purified preparations of pregnancy gonadotropin are unstable in aqueous solution. Because Katzman (10) had further observed that gonadotropic preparations from menopausal urine obtained by the benzoic acid method were inferior to similar preparations made by other methods, and had ascribed the inferiority of the first method to its acidity, attention was directed to the possible rôle of prolonged exposure to acid in the inactivations noted in our own preparations. The pH of our aqueous acetone extracts was invariably 4.5 to 5.0. To put the matter to experimental test, a number of purified preparations were deliberately exposed to the action of weak acid at 0–5° for varying periods of time, with the results shown in Table III. These results indicated that the purified hormone was even more labile than had hitherto been suspected, and suggested that the discrepancies noted in the extractions made with 30 per cent acetone might be obviated by closer attention to such details as the acidity of the extraction mixtures and the length of time during which the hormone was exposed to these acidities. We have, therefore, introduced several modifications which have resulted in a procedure that is superior to the earlier method and has been well standardized (see the accompanying diagram).


Early pregnancy urine, 10,000–50,000 m.e.d. per liter
Add benzoic acid
Filtrate
Benzoic acid ppt.
Extract with acetone
Filtrate
Residue (Product A), 30–50 m.e.d. per mg.
Extract with 50% ethanol at pH 6.0
Extract
Residue
Add 2 volumes ethanol
Filtrate
Ppt. (Product B), 200–500 m.e.d. per mg.
Extract with 50% ethanol at pH 4.8
Extract
Residue
Add 1 volume ethanol
Filtrate
Ppt. (Product C), 1000–3000 m.e.d. per mg.

Extraction of Product A with 50 Per Cent Ethanol—The procedure involves preliminary grinding of Product A (weight taken = x gm.) under 10 times its weight ($10x$ cc.) of cold 50 per cent aqueous ethanol, with careful addition of 2 per cent NH_3 in 50 per cent ethanol until a pH of 6.0 is reached.[5] The suspension is allowed to stand 24 hours at 0–5°, preferably in a wide vessel to produce a shallow layer, and is then centrifuged at high speed. The precipitate is washed several times by mechanical stirring with $2x$ cc. of 50 per cent ethanol. The combined alcoholic extracts (occasionally cloudy) are treated with 2 volumes of cold absolute ethanol to precipitate the hormone, and, after chilling for several hours, are centrifuged. The precipitate is washed with 85 per cent ethanol.

The material precipitated by alcohol is extracted three or four times by stirring for 30 minutes with $1x$ to $2x$ cc. of 0.3 M sodium acetate-acetic acid buffer in 50 per cent ethanol at pH 4.8.[5] After centrifugation, the combined acid-alcohol solutions are treated with an equal volume of cold absolute ethanol and chilled for several hours. The resulting precipitate obtained by centrifuging is successively washed with 75 and 95 per cent ethanol and finally several times with anhydrous acetone. It is occasionally necessary to add a trace of sodium acetate during the last washing. After thorough drying, a colorless amorphous product is obtained which consistently assays 1000 to 3000 minimal effective doses per mg. The yields are greatly improved over those observed with the 30 per cent acetone method. A summary of the results obtained is given in Table IV.

Further Purification Experiments—As was noted with the 30 per cent acetone products, no further purification of the fractions obtained with the 50 per cent ethanol method was observed after another extraction with the same solvent. Fractions of this order of activity were precipitated from aqueous solutions by uranyl acetate, tannic acid in the presence of acetic acid, and by complete saturation with ammonium sulfate. Little purification could be obtained with these reagents.

When aqueous solutions of similar fractions (3000 minimal effective doses per mg.) were shaken with chloroform at pH 4.5 to 5.0 (Sevag (11)), a small amount (1 to 5 per cent) of inert protein was precipitated. Upon removal of this impurity, the hormone was no longer readily precipitated with tannic acid in the presence of acetic acid, though it could be recovered if the solution was first acidified with sulfuric acid (12). Fractions similarly treated with chloroform were found to precipitate at pH 3.0 to 4.0 with iodine in potassium iodide, as described by Meyer (3). The hormone in preparations this active did not dialyze through cellophane membranes. Treatment with iodine in potassium iodide, tannic acid, or dialysis following removal of traces of protein with chloroform yielded preparations containing 4000 minimal effective doses per mg. and therefore appeared to effect a small purification of our ethanol extraction products.

---

[5] To determine the pH, a drop of the 50 per cent alcoholic solution was added to about 0.5 cc. of water on a spot plate and an appropriate indicator was added.

TABLE IV

Elution of Benzoic Acid Adsorbates with Aqueous Ethanol

Experiment No.	Starting material (Product A)			Final product (Product C)			Original activity recovered		
	Weight	Purity	Total activity	Weight	Purity	Total activity	In Product C	In residue from Product A	Total
	gm.	*m.e.d. per mg.*	*m.e.d. × 1000*	*mg.*	*m.e.d. × 1000 per mg.*	*m.e.d. × 1000*	*per cent*	*per cent*	*per cent*
1	5.00	33	165	147	1.0	147	90		90
2	0.28*	100	28	26	1.0	26	95		95
3	0.30*	100	30	20	1.0	20	65		65
4	5.00	33	165	102	2.0	204	100		100
5	10.00	33	330	168	2.0	336	100		100
6	25.00	25	625	325	1.0	325	50	45	95
7	25.00	25	625	367	1.0	367	60	45	105
8	21.00	25	525	171	1.5	257	50	40	90
9	18.60	50	930	146	3.0	438	45	55	100
10	17.00	50	850	338	3.0	1014	100		100

* Prepared by prolonged washing of benzoic acid precipitates followed by recovery from the cloudy supernatant acetone.

Preliminary cataphoresis experiments with a preparation assaying 500 minimal effective doses per mg. resulted in 2- to 3-fold purification when carried out at low temperatures. Because very little decomposition was observed under the conditions employed, a similar investigation of more active preparations is now under way.

Solubility of Purified Gonadotropin—Glycerol, ethylene glycol, ethylene chlorohydrin, and formamide were found to be good solvents for preparations assaying 500 to 2000 minimal effective doses per mg., while methyl, ethyl, and butyl alcohols, benzaldehyde, salicylaldehyde, furfuraldehyde, cresol, trimethylene glycol, ethylene glycol monoethyl ether, and β,β'-dihydroxyethyl ether dissolved relatively small amounts. Very little purification resulted from the use of any of the above reagents, nor were attempts to fractionate with aqueous ethyl alcohol or glycerol-ethyl alcohol mixtures more successful. It is of interest to note that the hormone was stable in glycerol solution when heated at 100° for 1 hour, while in formamide rapid inactivation took place at 0°. Ethylene glycol solutions were moderately stable, while the hormone appeared to undergo gradual inactivation in ethylene chlorohydrin.

Characteristics of Purified Gonadotropin—The preparations assaying 500 to 2000 minimal effective doses per mg. are colorless, amorphous powders containing approximately 10 per cent ash. They give the following positive qualitative tests: Molisch, biuret, ninhydrin, Millon, Pauly diazo, Sakaguchi, hexosamine, and ammoniacal $AgNO_3$ (slow reduction in the cold). The following tests are negative: Tollens' naphthoresorcinol, Seliwanoff, Hopkins-Cole. No precipitation from aqueous solutions is obtained with picric, picrolonic, flavianic, rufianic, trichloroacetic, or sulfosalicylic acids.

The preparations contain acetyl groups and hexosamine (Meyer (3))[6] as well as carbohydrate. While their activity appears to be somewhat greater than that of Meyer's preparations, their physical and chemical properties are similar to his and confirm his belief that the hormone is a mucoid. Tyrosine and arginine are found, while histidine and tryptophane are either absent or present only in traces. The hormone is inactivated by coupling with diazotized sulfanilic acid at pH 9.0 in the cold, as noted by Bischoff and Long (13). A preliminary report of the carbohydrate of our preparations has been made elsewhere (14). The details of this study, together with further chemical data, will appear in a later paper.

The applicability of the 50 per cent ethanol method to benzoic acid preparations of urine obtained at later periods of pregnancy has been examined in a preliminary way. Although further work is necessary, it appears that the method will also yield preparations active in 1000 minimal effective doses per mg. from urine obtained at such times.

---

[6] We wish to thank Dr. K. Meyer for his courtesy in determining the acetyl and hexosamine content of several of our preparations.

We are pleased to acknowledge the capable assistance of Miss Dorothy B. Hood.

SUMMARY

Two simple processes have been developed for obtaining highly active gonadotropic hormone preparations from pregnancy urine. Both involve preliminary adsorption of the hormone upon benzoic acid, and the recovery of the crude hormone, as described by Katzman and Doisy.

The first method consists of a single extraction of the crude hormone adsorption product with 30 per cent aqueous acetone. The preparations thereby obtained assay 500 to 2000 minimal effective doses per mg. on the postpartum rabbit.

The second method involves an extraction of the crude adsorption product with 50 per cent ethanol, at pH 6.0, and a reextraction of the derived product with the same solvent at pH 4.8. By this method preparations assaying 1000 to 3000 minimal effective doses per mg. are obtained. This method is well standardized for early pregnancy urine, from which good recoveries of hormone activity may be obtained.

Further purification of these products with tannic acid, iodine in potassium iodide, or dialysis, yields preparations containing 4000 minimal effective doses per mg.

Highly purified preparations contain a carbohydrate-polypeptide complex, and, as stated by Meyer, have the properties of a mucoid. Although stable in the dry state, the hormone in aqueous solution may undergo marked losses of biologic activity after 48 hours, even when kept in the cold.

BIBLIOGRAPHY

1. Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, **98**, 739 (1932).
2. Zondek, B., Scheibler, H., and Krabbe, W., *Biochem. Z.*, **258**, 102 (1933).
3. Meyer, K., in Kurzrok, R., The endocrines in obstetrics and gynecology, Baltimore, 115 (1937).

4. Browne, J. S. L., and Venning, E. M., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **116**, 18 (1936); *Lancet*, **2**, 1507 (1936).
5. Evans, H. M., Kohls, C. L., and Wonder, D. H., *J. Am. Med. Assn.*, **108**, 287 (1937).
6. Friedman, M. H., *J. Pharmacol. and Exp. Therap.*, **45**, 7 (1932); *Endocrinology*, in press.
7. Rowe, L. W., Simond, A., and Nelson, W. O., *J. Am. Pharm. Assn.*, **23**, 882 (1934).
8. Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, **106**, 125 (1934).
9. Gurin, S., Bachman, C., and Wilson, D. W., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. xlix (1938).
10. Katzman, P. A., *Endocrinology*, **21**, 89 (1937).
11. Sevag, M. G., *Biochem. Z.*, **273**, 419 (1934).
12. Lundin, H., and Schröderheim, J., *Biochem. Z.*, **238**, 1 (1931).
13. Bischoff, F., and Long, M. L., *J. Biol. Chem.*, **116**, 285 (1936).
14. Gurin, S., Bachman, C., and Wilson, D. W., *Science*, **89**, 62 (1939).

A NEW COUPLING COMPONENT FOR SULFANILAMIDE DETERMINATION*

By A. CALVIN BRATTON and E. K. MARSHALL, Jr.

With the Technical Assistance of Dorothea Babbitt and Alma R. Hendrickson

(From the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University, Baltimore)

(Received for publication, March 4, 1939)

The method proposed for the determination of sulfanilamide (1-3) has been widely used in estimating the drug in blood and urine both in experimental work and in controlling the dosage of the drug for patients. During the 2 years since the method has been in use, certain disadvantages have become apparent. The use of N, N-dimethyl-1-naphthylamine (dimethyl-α-naphthylamine) as the coupling component for the diazotized sulfanilamide is not entirely satisfactory on account of the necessity of a catalyst for rapid development of color in dilute solutions, the need of a large excess of the reagent, and the necessity of a certain amount of alcohol to keep the resultant azo dye in solution. A coupling component which can be obtained in the form of a crystalline salt of reproducible composition and which gives a soluble azo dye in acid solution appeared desirable. The other defect which was discovered in the method was that certain samples of dimethyl-α-naphthylamine did not give complete recovery of sulfanilamide added to normal blood. This was found to be due to the salts (mainly chloride) present in the blood filtrate catalyzing the destruction of the azo dye by the excess nitrite.

Modifications of our method by various authors offer no real advantages. In the main, these procedures have consisted in altering the amount of blood and reagents used (4), a purification of the coupling component (5), or a restatement of slight modifica-

---

* This investigation has been aided by a grant from the John and Mary R. Markle Foundation.

tions already described by the author (6, 7). Two important improvements in the method were described about a year ago (8); namely, the destruction of excess nitrite by ammonium sulfamate and the buffering of the diazotized solution before coupling with dimethyl-α-naphthylamine. The destruction of excess nitrite, by preventing the formation of nitroso compounds, allows the use of a much wider variety of coupling components than is otherwise possible. Previously, but unknown to us at the time, Hecht (9) determined N^4-sulfanilyl-N^1, N^1-dimethylsulfanilamide in urine and blood by coupling the diazotized compound with N-ethyl-1-naphthylamine after destruction of the excess nitrite with sulfamic acid or urea.

We decided that the ideal coupling agent for determination of sulfanilamide should exhibit rapidity of coupling, sensitivity, purity, and reproducibility, be unaffected in rapidity of coupling by changes of pH from 1 to 2, and that the azo dye formed should be acid-soluble and not affected in color by pH changes from 1 to 2. A number of compounds which appeared to be promising in these respects and which couple in acid solution[1] have been examined.

The rapidity of coupling (speed) was noted for diazotized 0.1 and 0.01 mg. per cent solutions of sulfanilamide buffered to pH 1.3; also the influence of pH on the rapidity of coupling with a 1 mg. per cent solution, the sensitivity with a 0.01 mg. per cent solution, the solubility of the azo dye with a 10 mg. per cent solution, and the effect of pH on the color of the dye in the same solutions used for determining the effect of pH on speed. Trichloroacetic acid was used for acidification, excess nitrite was destroyed with ammonium sulfamate, and pH was varied by adding excess acid or sodium dihydrogen phosphate. In Table I are summarized these preliminary tests on seventeen compounds. Two aqueous solutions of each coupling component were used, one of such a strength that 10 moles per mole of diazonium salt were used for the 10 mg. per cent solution of sulfanilamide and the other one-tenth as strong for use with the other dilutions of the drug. A minimal quantity of hydrochloric acid was used to dissolve coupling Compounds 1 to 3, 6 to 9, and 13; sodium hydroxide was employed on Compounds 4, 10 to 12, 15, and 16; alcohol was necessary for Compound 17. Coupling Compounds 2 to 4, 7, and 10 to 17 were obtained from E. I. du Pont de Nemours and Company, Inc., through the courtesy of Dr. H. A. Lubs and Dr. D. E. Kvalnes. Compounds 1,[2] 2, 4, 7, 8, 15, and 17 were prepared or purified from the commercial base or salt before use, Compounds 1, 6, 8, and 9 were from the Eastman Kodak Company, and Compound 5 was synthesized by us.

---

[1] Coupling in alkaline solution has certain disadvantages which have been cited (3).

On the basis of rapidity and sensitivity only five compounds (Nos. 2, 3, 5, 7, and 8) offer improvement over N,N-dimethyl-1-naphthylamine (No. 1). Of these, Compounds 7 and 8 are eliminated because they yield precipitates with diazotized 1 mg. per cent sulfanilamide. Compound 3 is eliminated because of the considerable influence of pH on rapidity of coupling, leaving only Compounds 2 and 5 for consideration. Compound 2 is very difficult to purify, and possesses no definite physical properties to aid in its characterization. It is to be expected that different batches of the compound would show considerable variation in purity, and the lack of reproducibility would render it unsuitable for use in the method. Compound 5, N-(1-naphthyl)ethylenediamine dihydrochloride may be readily prepared in a state of high purity. Its coupling is very rapid and uninfluenced by pH in the range of 1 to 2. This range of pH has no effect on the color of the dye, and the dye is more soluble in this range than that from any other coupler we have examined.

Preparation and Purification of N-(1-Naphthyl)Ethylenediamine Dihydrochloride

No adequate chemical description was given by Newman (10, 11) who first prepared this compound through the Gabriel synthesis. Therefore, the following modifications of Newman's synthesis and a rather detailed characterization of the compound seem justified.

β-(1-Naphthylamino)Ethylphthalimide—This was prepared by essentially the procedure of Newman, except that 2.5 moles of 1-naphthylamine to 1 mole of β-bromoethylphthalimide were used, and the product was recrystallized from glacial acetic acid.

---

[2] This sample of dimethyl-α-naphthylamine was a pure sample which had been aerated 30 minutes at 267° (3), and distilled at reduced pressure.

TABLE I

Suitability of Some Coupling Components for Sulfanilamide Determination

Compound	Speed for 0.01 and 0.1 mg. per cent solutions at pH 1.3	Color of dye, 10 mg. per cent solution	Influence on coupling speed 1 mg. per cent solution			Sensitivity, 0.01 mg. per cent solution	Ppt., 10 mg. per cent solution	Effect on dye color, 1 mg. per cent solution		
			pH 1.0	pH 1.3	pH 1.6			pH 1.0	pH 1.3	pH 1.6
1. N,N-Dimethyl-1-naphthylamine	+	Purple-red	+	++	++	+	+++	++	++	++
2. N-(1-Naphthyl)glucamine	+++	"	++++	++++	++++	+	+	++++	++++	++++
3. N,N-Di-(hydroxyethyl)-1-naphthylamine	++	Violet	+	++++	++++	+	++	++++	++++	++++
4. Sulfonated N-ethyl-1-naphthylamine	+	Violet-red	+	+	+	+	+++	+	+++	++++
5. N-(1-Naphthyl)ethylenediamine dihydrochloride	+++	Purple-red	++++	++++	++++	+	0	++++	++++	++++
6. 2-Naphthylamine	0	Orange				+	+++			
7. N-Ethyl-1-naphthylamine hydrochloride	++	Purple	−	−	−	+	+++			
8. N-Methyl-1-naphthylamine acid sulfate	++	Violet	−	−	−	+	+++			
9. 1-Naphthylamine	+	Violet-red	−	−	−	+	+++			
10. 1-Hydroxyethylamino-5-naphthol	+	Purple-red				0	+++			
11. 1-Amino-5-naphthol	+	Violet				0	+++			
12. Phenyl J acid (N-phenyl-2-amino-5-naphthol-7-sulfonic acid)	+	Orange-red				0	++			

13. N,N-Diethyl-1-naphthylamine	0	Red				0	+			
14. H acid (1-amino-8-naphthol-3,6-disulfonic acid)	+	"				0	++			
15. Phenyl peri acid (phenyl-1-naphthylamine-8-sulfonic acid)	++	Violet	–	–	–	0	+++			
16. Tolyl peri acid (*p*-tolyl-1-naphthylamine-8-sulfonic acid)	+	Purple				0	+			
17. N-Phenyl-1-naphthylamine	–	"				–	+++			

0 = color no greater than blank; – = precipitate formation; + = degree of color, speed, *etc.*

The yield was 68 to 78 per cent of theory. If desired, half the 1-naphthylamine in the synthesis may be replaced by an equivalent amount of sodium bicarbonate, resulting in a yield of 55 to 60 per cent.

The product is extensively soluble in hot glacial acetic acid, benzene, and ethyl acetate, moderately in alcohol. It is moderately soluble in cold ethyl acetate and benzene, fairly soluble in alcohol, and slightly soluble in ether. It crystallizes from acetic acid in greenish yellow, thin, irregular plates, m. p. 165.3–165.7°. (A second recrystallization from this solvent with the addition of activated charcoal yields a golden yellow product.)

Hydrolysis of β-(1-Naphthylamino)Ethylphthalimide—The use of ordinary hydrolytic agents, even fuming hydrochloric acid as employed by Newman, results in poor yields. Either of the following two methods may be employed.

(*a*) *By Fused Sodium Hydroxide*—β-(1-Naphthylamino)ethylphthalimide was ground intimately with an equal weight of solid sodium hydroxide, and the mixture was distilled rapidly at 10 to 20 mm. pressure. The mass melted at 160°, an oily yellow distillate passed over at 200–300°, and the distillation was discontinued when the residue began to char at 340°. The distillate was extracted with the calculated amount of 0.05 N hydrochloric acid (stronger acid yielded a turbid extract), and after the material was decolorized with charcoal, the base was liberated with excess sodium hydroxide, taken up in benzene, and dried over solid sodium hydroxide. Upon distilling off the solvent and taking up the base in a little alcohol, the calculated amount of hot alcoholic picric acid was added and the solution was cooled. The red-brown picrate was recrystallized from 6 N acetic acid (or 95 per cent alcohol), and precipitated as tiny red octahedra in 40 to 70 per cent yield. Its melting point is not sharp, and depends upon the rate of heating: placed at 222° into a fairly rapidly rising bath, m.p. 227–228° with decomposition; in a more slowly heated bath, m.p. 225–226° with decomposition.

$C_{18}H_{17}N_5O_7$. Calculated. C 52.02, H 4.13, N 16.87
Found. " 52.21, " 4.12, " 16.82

Smaller still charges (8 to 10 gm.) gave higher yields, probably due to the lower heat gradient in the mass. Larger batches (60 to 70 gm.) gave lower yields because of a side reaction involving scission of the side chain to yield 1-naphthylamine, whose picrate may be obtained from the alcoholic picric acid mother liquor by addition of 5 volumes of water. In one experiment with 0.0994 mole of β-(1-naphthylamino)ethylphthalimide, 0.0398 mole of N-(1-naphthyl)ethylenediamine picrate and 0.0124 mole of 1-naphthylamine picrate were obtained.

Liberation of Base—The picrate was suspended in warm water, treated with sodium hydroxide in slight excess, and the liberated N-(1-naphthyl)ethylenediamine was taken up in benzene and dried over solid sodium hydroxide. The dihydrochloride may be prepared by bubbling dry hydrogen chloride into the benzene solution, or the solvent may be evaporated and the base distilled under reduced pressure.

(*b*) *By Hydrazine*—Refluxing β-(1-naphthylamino)ethylphthalimide in alcoholic suspension for 2 hours with hydrazine hydrate, followed by addition of excess hydrochloric acid and continuation of refluxing for 1 hour, results in an 85 to 90 per cent yield of fairly pure N-(1-naphthyl)ethylenediamine dihydrochloride. The procedure was essentially that of the general method of Ing and Manske (12). Substitution of the hydrazine hydrate by an equivalent amount of hydrazine acid sulfate, sodium carbonate, and a minimal amount of water gave a more nearly pure product in slightly lower yield (70 to 80 per cent).

Properties of N-(1-Naphthyl)Ethylenediamine—The base is a straw-yellow, viscous liquid with an odor resembling that of the alkyl naphthylamines; the boiling point is 204° at 9 mm., about 320° with decomposition at 760 mm.; $n_D^{25} = 1.6648$; $d_4^{25} = 1.114$. The solubility in water is about 0.2 gm. in 100 cc. at 25°, more soluble in hot than in cold water; the pH of a saturated aqueous solution is 10.5. The base is readily soluble in the common organic solvents, except petroleum ether. It distils very poorly with steam, even from concentrated alkali.

Salts of Base—The dihydrochloride is prepared by introducing dry hydrogen chloride into a solution of the base in benzene or ether, or by dissolving the base in excess hot 6 N hydrochloric acid. Recrystallized from 6 N hydrochloric acid, it precipitates in long colorless hexagonal prisms. Use of activated charcoal is of advantage in obtaining a perfectly white preparation. We were unable to dry the dihydrochloride without loss of a little hydrogen chloride, which resulted in a poor analysis. A mixture of the mono- and dihydrochlorides is perfectly satisfactory for use in the method, but it is, of course, advantageous to strive for a pure dihydrochloride in order that the melting point may serve in identification and control of purity. For this reason, the excess mother liquor should be removed by pressing between filter paper and the bulk of the remaining water should be removed *in vacuo* or by air drying. The last traces of water are removed by heating briefly at 110° and transferring while still warm to a vacuum desiccator. M.p. (placed at 184° in a fairly rapidly rising bath) 188–190°. If the dihydrochloride is distilled at reduced pressure, a product is obtained which melts at 231–232° with slight decomposition, and is probably the monohydrochloride. The dihydrochloride is easily soluble in 95 per cent alcohol, dilute hydrochloric acid, and hot water; it is rather difficultly soluble in cold water, acetone, and absolute alcohol.

$C_{12}H_{16}N_2Cl_2$. Calculated. C 55.58, H 6.23, N 10.82, Cl 27.37
Found. " 55.52, " 6.20, " 10.41, " 26.81

Prolonged drying of the analytical sample at 110° raised the determined nitrogen to 11.60 per cent and lowered the halogen to 18.76 per cent.

The zinc chloride and mercuric chloride salts and the acid sulfate were prepared but the first is too soluble, and the melting points of the latter two too high, to be of use in purification or identification of the base.

Determination of Sulfanilamide in Blood and Urine

Reagents[3]—

1. A solution of trichloroacetic acid containing 15 gm. dissolved in water and diluted to 100 cc.
2. A 0.1 per cent solution of sodium nitrite.
3. An aqueous solution of N-(1-naphthyl)ethylenediamine dihydrochloride containing 100 mg. per 100 cc. This solution should be kept in a dark colored bottle.
4. A solution of saponin containing 0.5 gm. per liter.

---

[3] The reagents can be obtained from LaMotte Chemical Products Company, Baltimore.

5. 4 N hydrochloric acid.

6. A solution of ammonium sulfamate, containing 0.5 gm. per 100 cc.

7. A stock solution of sulfanilamide in water containing 200 mg. per liter. This solution can be kept for several months in the ice box. The most convenient standards to prepare from the stock solution are 1, 0.5, and 0.2 mg. per cent. To prepare these 5, 2.5, and 1 cc. of the stock solution plus 18 cc. of the 15 per cent solution of trichloroacetic acid are diluted to 100 cc.

Procedure for Blood[4]—2 cc. of oxalated blood are measured into a flask and diluted with 30 cc. of saponin solution, and after 1 or 2 minutes precipitated with 8 cc. of the solution of trichloroacetic acid. The free sulfanilamide is determined in the filtrate as follows: 1 cc. of the sodium nitrite solution is added to 10 cc. of the filtrate. After 3 minutes standing, 1 cc. of the sulfamate solution is added, and after 2 minutes standing, 1 cc. of the solution of N-(1-naphthyl)ethylenediamine dihydrochloride is added. The unknown is compared with an appropriate standard which has been treated as above. This comparison can be made immediately and no change in color is observed for 1 hour or more. To determine the total sulfanilamide, 10 cc. of the filtrate are treated with 0.5 cc. of 4 N hydrochloric acid, heated in a boiling water bath for 1 hour, cooled, and the volume adjusted to 10 cc. The subsequent procedure is as stated above for determining free sulfanilamide.

Procedure for Urine—Protein-free urine is diluted to contain about 1 to 2 mg. per cent of sulfanilamide and 50 cc. of the diluted urine plus 5 cc. of the 4 N hydrochloric acid are diluted to 100 cc. 10 cc. of the product of this second dilution are treated as a blood filtrate for free sulfanilamide, and 10 cc. heated without further addition of acid for total sulfanilamide. If the urine contains protein, it is diluted and treated by the procedure for blood.

Photoelectric Colorimeter—When a photoelectric colorimeter is available, dilutions of blood of 1:50 or 1:100 can be used. The blood is diluted with water (saponin is unnecessary), allowed to stand a few minutes, and precipitated with trichloroacetic acid solution, with a volume which is one-fifth that of the final mixture.

---

[4] Sample and reagent volumes can be proportionately reduced to give the minimal amount of filtrate necessary for an accurate color comparison.

This allows the use of 0.1 or 0.2 cc. samples of blood which are measured with washout pipettes. Determinations on urine or other body fluids are easily made after appropriate dilution. The reagent blank on distilled water is quite low, but increases with time if the solution is left in the light. For this reason, solutions to be read in the photoelectric colorimeter should be protected from light unless the reading is made immediately. Some reaction occurs between the trichloroacetic acid and the N-(1-naphthyl)ethylenediamine, since solutions acidified with hydrochloric acid do not show an increased color on exposure to light. The blood blank is extremely low and negligible for most purposes. With a 1:50 dilution of human blood the correction due to the blood blank varies from 0 to 0.03 mg. per cent. This blank can be easily determined by performing an analysis as usual except that water is substituted for the sodium nitrite solution. When small concentrations of sulfanilamide are to be determined or when a foreign dye such as prontosil or neoprontosil is present, this procedure is quite useful. The color of the normal urinary pigments can be conveniently corrected for by the same procedure.

When only a very small amount of blood is available, as in the case of small animals such as mice, a determination can be made with considerable accuracy on 0.02 cc. The adaptation is essentially that described by Marshall and Cutting (13), the dilution of blood being 1:200 or 1:400, depending on the concentration of sulfanilamide present. The proportion of reagents used is the same as in the other adaptations of the method. Centrifugation before filtration of the protein precipitate is useful in securing the maximum amount of filtrate.[5]

In using a photoelectric colorimeter a filter is essential. With dimethyl-α-naphthylamine the peak of the absorption of the azo dye formed occurs at 530 mμ (14). When N-(1-naphthyl)-ethylenediamine is used, the peak of absorption is shifted to 545 mμ, and the dyes from sulfapyridine and N^1-ethanolsulfanilamide show the same absorption peak. In Fig. 1 is reproduced an absorption curve of the azo dye from sulfanilamide. We wish to thank Dr. Elizabeth E. Painter of the Department of Physiology, Columbia University, New York, for these absorption data.

---

[5] For these dilutions (1 : 200 or 1 : 400, a photoelectric colorimeter of high sensitivity, designed and constructed by Dr. Morris Rosenfeld of this department, was used. A description of this instrument will be published.

Blood Dilution and Recovery—When a 1:4 dilution of blood is used as suggested in Fuller's method (15) and in Proom's (6) adaptation of our method, a result 10 per cent too low may be obtained. Sulfanilamide was added to three samples of mixed human blood to give about 10 mg. per cent, and determinations made with various dilutions for precipitation. The filtrates were diluted when necessary to read with the photoelectric colorimeter. Average percentage recoveries were for the 1:4 dilution 90.0, for the 1:10 dilution 94.3, for the 1:20 dilution 96.9, and for the 1:50 dilution 99.5. Determinations on other samples of blood with added sulfanilamide gave similar recoveries for the 1:20 and 1:50 dilutions.

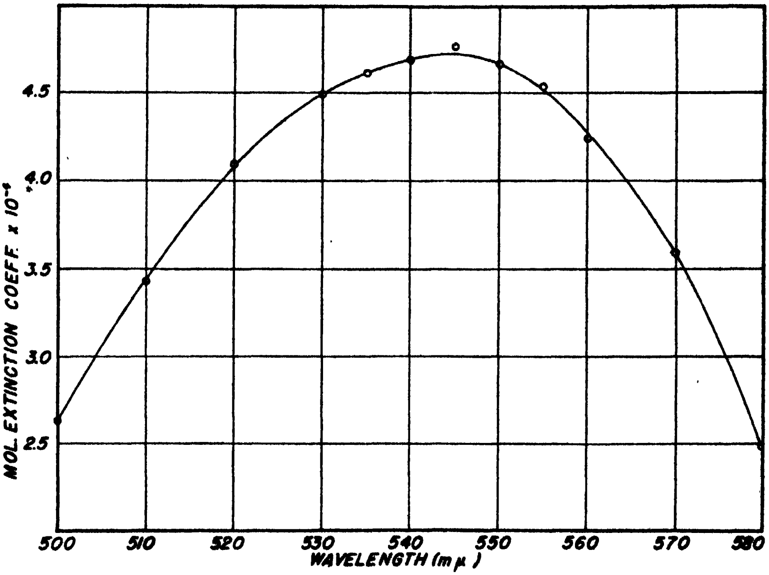


FIG. 1. Absorption curve of dye from coupling diazotized sulfanilamide with N-(1-naphthyl)ethylenediamine.

With acetylsulfanilamide recovery is not complete in a 1:20 dilution of blood (about 90 per cent) but is complete in a 1:50 dilution. A series of eight bloods to which varying amounts of acetylsulfanilamide were added gave recovery of 97.6 per cent

(96.2 to 99.2) with the dimethyl-α-naphthylamine reagent in a 1:50 dilution. Since experiments show that solutions of pure acetylsulfanilamide give theoretical results when hydrolyzed with hydrochloric acid and estimated by use of the new coupling component, no recoveries of the acetyl compound with the new reagent were made.

The accidental errors of the method can be best illustrated by some determinations made with the photoelectric colorimeter. Sulfanilamide was added to a sample of mixed human blood and fifteen determinations were made on the sample, 0.5 cc. of blood (measured with a syringe pipette) being used each time in a 1:50 dilution. The mean value in mg. per cent was 4.888 ± 0.039 with a maximum deviation of 0.113.

Protein Precipitation—Precipitation of the blood proteins with trichloroacetic acid has been adopted in place of *p*-toluenesulfonic acid for the following reasons: trichloroacetic acid of constant quality and purity can be obtained much more readily than can toluenesulfonic acid, no difficulty is experienced in determining total sulfanilamide in a trichloroacetic acid blood filtrate when the excess nitrite is destroyed (8), and the blood blank given with trichloroacetic acid is much less than that obtained when toluenesulfonic acid is used. The last mentioned advantage is of great importance when a photoelectric colorimeter is used, as the blank obtained on human blood with trichloroacetic acid can usually be neglected but must be taken into account when a toluenesulfonic acid filtrate is used.

Body Fluids Other Than Blood and Urine—No difficulty has arisen in estimating sulfanilamide and its acetyl derivative in other body fluids by the same procedure as used for blood. When tissues are to be analyzed, it appears desirable to extract the ground tissue in a Soxhlet apparatus with a limited amount of alcohol, dilute an aliquot portion of the extract with water, and proceed as in blood, with a photoelectric colorimeter. This is a simpler and less laborious method than the one previously used for tissues (16).

Sulfanilamide Derivatives—As previously indicated (3), our method can be used for determining diazotizable primary aryl amines containing either a free amino group or a blocked amino group which can be freed by hydrolysis. In the limited experience which we have had in applying our method to the estimation of compounds other than sulfanilamide, three points of importance can be mentioned. With a very difficultly soluble substance, the recovery in the blood filtrate is generally not quantitative unless high dilutions (1:100 or greater) are used, so that either one must use such dilution or must resort to the original alcohol precipitation method (1). In the latter case, ammonium sulfamate is used to destroy excess nitrite and N-(1-naphthyl)ethylenediamine dihydrochloride is used as a coupling component. With certain derivatives, the azo dyes formed will not be acid-soluble and a certain amount of alcohol must be added with or just before the coupling component. With some substances buffering is necessary to obtain sufficient speed of coupling (*e.g.*, aniline).

In the determination of sulfapyridine (2-(sulfanilamido)-pyridine) with the present method, the following may be mentioned. To a sample of mixed human blood sulfapyridine was added to make about 10 mg. per cent. Precipitation in a 1:4 dilution gave 80.8 per cent recovery, in a 1:20 dilution 93.7 per cent, and in a 1:50 dilution 99.4 per cent. A number of other experiments indicate incomplete recovery (average 91 per cent) in 1:20 dilution with 5 to 10 mg. per cent in blood, but essentially complete with values below 5 mg. per cent. With a 1:50 or greater dilution, recovery is quantitative.

DISCUSSION

We have already discussed some of the so called modifications of our method which have been proposed. It remains to mention briefly other methods which have been suggested for the determination of sulfanilamide or allied compounds. Kühnau (17) has described a method for estimating N^4-sulfanilyl-N^1, N^1-dimethylsulfanilamide by the color produced with dimethylaminobenzaldehyde, and Schmidt (18) one for sulfanilamide, with the color produced by sodium β-naphthoquinone-4-sulfonate. We have had no experience with either of these. Scudi (19) has described a diazotization procedure, followed by neutral coupling with chromotropic acid, while Doble and Geiger (20) used diphenylamine as an acid coupling agent. Neither of these latter methods appears to be as satisfactory as the method we have described.

With a new compound, a few preliminary trials should indicate what slight modification of the method, if any, is necessary to determine it accurately. However, we must caution investigators against accepting results with a new compound until control recoveries from blood and urine have been made.

SUMMARY

In the determination of sulfanilamide by diazotization and coupling in acid solution, the use of N-(1-naphthyl)ethylenediamine dihydrochloride offers the following advantages over N,N-dimethyl-1-naphthylamine (dimethyl-α-naphthylamine): (1) reproducibility and purity, (2) greater rapidity of coupling, (3) increased sensitivity, (4) elimination of buffer, and (5) increased acid solubility of the azo dye formed. An improved synthesis and a complete characterization of the new coupling component is presented. Slight modification of previous technique and application to other primary aryl amines are described.

BIBLIOGRAPHY

1. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C., *J. Am. Med. Assn.*, **108**, 953 (1937).
2. Marshall, E. K., Jr., *Proc. Soc. Exp. Biol. and Med.*, **36**, 422 (1937).
3. Marshall, E. K., Jr., *J. Biol. Chem.*, **122**, 263 (1937-38).
4. MacLachlan, E. A., Carey, B. W., and Butler, A. M., *J. Lab. and Clin. Med.*, **23**, 1273 (1938).
5. Stevens, A. N., and Hughes, E. J., *J. Am. Pharm. Assn.*, **27**, 36 (1938).
6. Proom, H., *Lancet*, **1**, 260 (1938).
7. Kamlet, J., *J. Lab. and Clin. Med.*, **23**, 1101 (1938).
8. Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, **88**, 85 (1938).
9. Hecht, G., *Dermat. Woch.*, **106**, 20 (1938).
10. Newman, H. E., *Ber. chem. Ges.*, **24**, 2199 (1891).
11. British patent 247,717, June 29, 1925.
12. Ing, H. R., and Manske, R. H. F., *J. Chem. Soc.*, 2348 (1926).
13. Marshall, E. K., Jr., and Cutting, W. C., *Bull. Johns Hopkins Hosp.*, **63**, 328 (1938).
14. Gregersen, M. I., and Painter, E. E., *Am. J. Physiol.*, **123**, 83 (1938).
15. Fuller, A. T., *Lancet*, **1**, 194 (1937).
16. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C., *J. Pharmacol. and Exp. Therap.*, **61**, 196 (1937).
17. Kühnau, W. W., *Klin. Woch.*, **17**, 116 (1938).
18. Schmidt, E. G., *J. Biol. Chem.*, **122**, 757 (1937-38).
19. Scudi, J. V., *J. Biol. Chem.*, **122**, 539 (1937-38).
20. Doble, J., and Geiger, J. C., *J. Lab. and Clin. Med.*, **23**, 651 (1938).

THE SURVIVAL OF CATHEPSIN IN AUTOLYSIS*

By HOWARD EDER, H. C. BRADLEY, and SAMUEL BELFER

(From the Department of Physiological Chemistry, University of Wisconsin, Madison)

(Received for publication, February 3, 1939)

One of the fundamental observations on tissue autolysis was that acid increased not only the speed of hydrolysis, as indicated by the slope of the digestion curve, but also the amount of hydrolysis, as indicated by the height attained at equilibrium. Thus in a liver pulp at pH 7.5 about 25 per cent of the total protein is dissolved. At pH 4 about 90 per cent goes into solution, and the residue is made up of the connective tissue stroma of the gland, which is not digested by cathepsin under any circumstances. We have interpreted the small digestion at pH 7.5 as indicating the relatively small mass of substrate available at that pH, while the complete proteolysis at pH 4 indicates that all the tissue proteins are available, except the stroma. The actual substrate we believe to be the protein cation (1–4), or possibly the isoelectric protein molecule (5). The validity of the interpretation, however, rests upon the maintained activity of cathepsin.

Very few data exist on the survival of cathepsin. In our earlier studies it was found active after 3 weeks at the reaction of the liver pulp, at about pH 6.2. If such a digest were made slightly acid, digestion was at once resumed (6). At pH 2.5 cathepsin is rather rapidly destroyed (7). This observation has been confirmed recently by Anson (8), using a purified cathepsin preparation and hemoglobin as substrate. He also showed that cysteine prolongs the activity of cathepsin in acid solutions. The fact, as also shown by Anson, that gelatin is not digested by cathepsin but by other members of the proteolytic system present in tissues

---

* This work was facilitated by the assistance of Works Progress Administration funds of the University of Wisconsin.

invalidates certain of our own earlier conclusions on the survival of cathepsin, and at the same time indicates that much of the European work upon that enzyme must be revised (5, 9–11).

Since interpretations of autolysis figures obtained on whole tissue digests have been based upon the assumption of enzyme survival, it seems desirable to reinvestigate this point. It is clear that the rapid destruction of the enzyme may be quite as important in producing an apparent equilibrium in a digest as lack of substrate.

Procedure

Freshly killed pig liver was ground to the consistency of thick cream, and diluted to make 20 per cent of the final digest, in which 5 per cent by volume of toluene was present. A liter of such a mixture constituted one of a series of primary digests, set approximately at the following pH levels: 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9. The pH levels of these primary digests were checked at frequent intervals and readjusted when necessary so as to maintain the original H ion concentration throughout autolysis. Primary digestion is represented in Figs. 1 to 5 by the continuous curves.

Secondary digests were made up by removing 95 cc. of the primary digests and adding 5 cc. of 13 per cent hemoglobin solution. All secondary digests were adjusted to pH 4, and maintained at that value throughout the course of the secondary digestion. An initial secondary digest was made up at once; the others at intervals of 1, 3, 5, 10, 20, and 30 days. Secondary digests are represented by broken curves.

Samples were taken at the start and at intervals thereafter and precipitated with an equal volume of 10 per cent trichloroacetic acid. Soluble nitrogen and reactive tyrosine were determined in the protein-free filtrates as the measure of digestion. Only the data on soluble nitrogen are presented, since they give the most direct evidence of digestion. The tyrosine figures parallel closely those of the nitrogen and confirm all of the important features of the latter.

It will be noted that all primary digests were identical in their gross substrate content, and all of the secondary digests were identical both in substrate and the pH at which they were maintained. Primary and secondary digests differed only in the substitution of 5 cc. of 13 per cent hemoglobin for 5 cc. of 20 per cent liver pulp per 100 cc. of total digest, and in the pH.

We have selected a few typical curves to illustrate the general conclusions which can be drawn from this study.

Fig. 1 represents the extent of digestion in the primary series at various times, as related to pH. It is a typical picture of catheptic activity of the liver, and with minor changes in the shape of the curves represents other tissues as well. It is interesting to note the gradual shift of the maximum from left to right as digestion continues, and the broadening of the zone of maximum autolysis; the rapid falling off of autolysis on the acid side compared to the gradual decrease on the alkaline side of the maximum. The data will show that the failure to digest to the maximum on the acid side is due in part at least to enzyme destruction, which at pH 2 is immediate. On the alkaline side destruction is relatively slow and is of less importance in determining the shape of the curve.

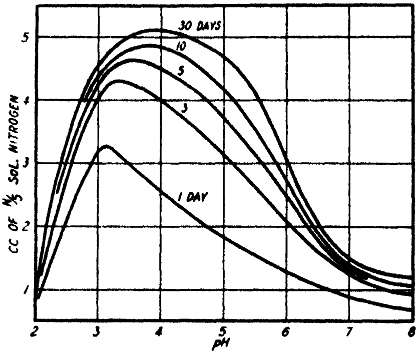


FIG. 1. Liver autolysis related to pH

At pH 2.42 cathepsin grows inactive fairly rapidly (Fig. 2). There is some activity at the end of 3 days; none at the end of 5. Leveling off of the primary digestion at a height below that attained in less acid autolysis appears to be determined primarily by the loss of enzyme activity.

Autolysis at pH 3.0 (*Fig. 3*)—Digestion starts rapidly at this pH, and levels off 50 per cent above the autolysis at pH 2.5, but below the optimum shown in Fig. 4. The secondary digests show gradual loss of enzyme activity, as indicated by the failure of each succeeding secondary digest to reach the height attained by the one before it. There is very little residual cathepsin at 20 days. Equilibrium here appears to be due to the inhibiting effects of the cleavage products upon the decreasing mass of enzyme, which thus becomes finally the limiting factor.

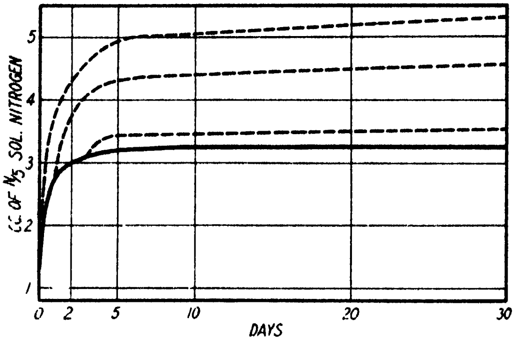


Fig. 2. Cathepsin survival at pH 2.42

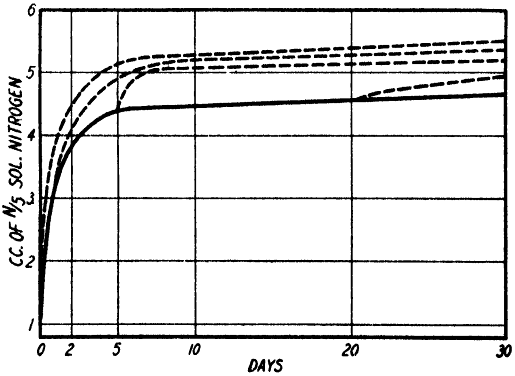


Fig. 3. Cathepsin survival at pH 3.07

Autolysis at pH 4 and 5—Fig. 4, pH 3.85, represents autolysis in the optimum range in which proteolysis is eventually complete, and the material remaining undigested consists of the structural proteins of the gland which are resistant to cathepsin under all conditions. The enzyme is abundant at the end of 30 days, though some loss of activity has occurred. During the first 10 days autolysis, the secondary curves show evidence of enzyme increase as a result of the process. This production of more enzyme during autolysis appears to be in harmony with the observations of Willstätter (12, 13), who described cathepsin in the tissues in two forms, "lyo" and "desmo." The "lyo" form is dispersed and may be extracted from the tissue at once. The "desmo" form is fixed in the tissue, but by autolysis is eventually dispersed, and is then indistinguishable from the "lyo" form. The final leveling off of the primary digest at this pH is attributable to the complete solution of all the liver proteins present, with the exception of the stroma residues.

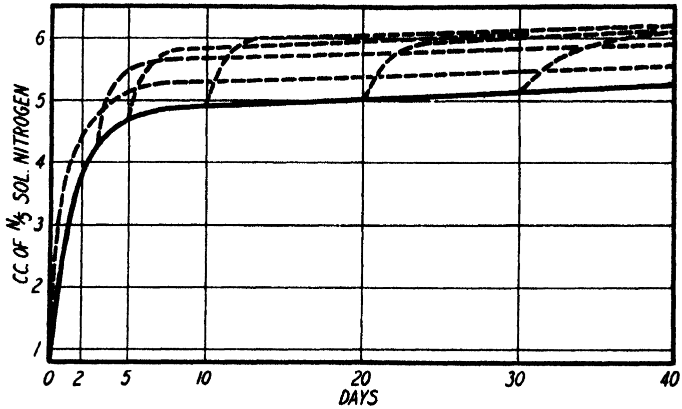


Fig. 4. Cathepsin survival at pH 3.85

The series at pH 5 presents much the same features as that at pH 4. Survival is rather better than in the more acid digest. The same increase of cathepsin is found as autolysis proceeds. The initial rate of digestion is considerably slower at pH 5 than at pH 4. This is evident in the 24 hour curve in Fig. 1. When autolysis is initiated at pH 4 and 5, the mass of available substrate in each is very large, and nearly equal. There is nearly twice as much proteolysis, however, in 24 hours at pH 4 as at 5. This would suggest that the enzyme itself is more active at pH 4, and so far as we are aware is the only evidence thus far reported which indicates that the H ion concentration affects the activity of the enzyme as well as the availability of the protein. We shall expect to report in more detail upon this point later.

Autolysis at pH 6 to 9—Fig. 5 represents digestion at pH 6. With increasing alkalinity its general features remain the same but with increasing evidence of enzyme destruction. At pH 7 there is considerable activity remaining at the end of 30 days, though less than in the pH 6 series. At pH 8 some cathepsin is still present at the end of 20 days; none by the 30th. At pH 9 cathepsin is present for 10 days at least although there is no digestion in the primary digest after the 1st day.

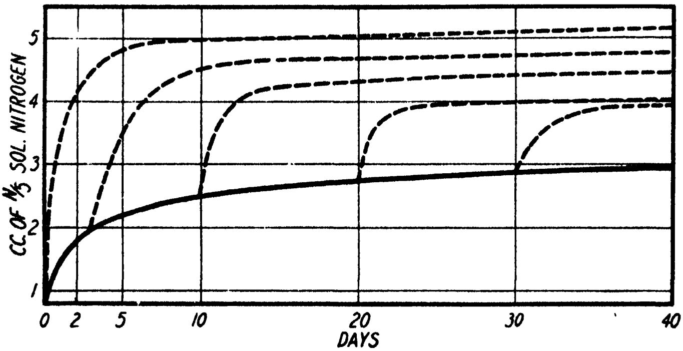


FIG. 5. Cathepsin survival at pH 6.12

DISCUSSION

The experiments reported indicate that cathepsin survives for long periods in the reaction zone for optimum activity, or between pH 3.5 and 5. At higher acidities the enzyme is destroyed in proportion to the acidity, and the enzyme itself becomes the limiting factor which determines the height of digestion attained. In autolyses more alkaline than pH 5 the enzyme is slowly destroyed, but until high alkalinities are reached, the diminishing mass of enzyme does not appear to be the factor which limits digestion. Lack of available substrate, that is when the protein exists chiefly in the form of base protein salt or the protein anion, is the explanation which has been given to this phenomenon (3). It is also probable that the activity of the enzyme itself is conditioned by the pH of the medium, in the sense that at pH 7 there is less active cathepsin present than at pH 4. The smaller the mass of enzyme present, the smaller the mass of cleavage products required to inhibit its activity. So far as we are aware there are no data which yet clearly indicate whether the effect of increased H ion activates the enzyme or the substrate or both.

SUMMARY

1. Reactions more acid than pH 3 lead to rapid destruction of liver cathepsin. At pH 2 the enzyme is destroyed at once.

2. From pH 3.5 to 5 cathepsin maintains its activity for several weeks.

3. In solutions more alkaline than pH 5 there is gradual inactivation in proportion to the alkalinity. Even at pH 8 some activity remains at the end of 10 days.

4. Cessation of proteolysis in digests more acid than 3.5 is determined by failure of the enzyme rather than lack of substrate. At the optimum pH zone digestion in the sense of proteolysis is complete and includes all the liver proteins except connective tissue stroma.

5. On the alkaline side of pH 4, equilibria appear to be determined by the mass of available substrate, though reduction in enzyme activity by low H ion concentration has not yet been adequately ruled out.

BIBLIOGRAPHY

1. Bradley, H. C., *J. Biol. Chem.*, **22**, 113 (1915).
2. Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, **25**, 261 (1916).
3. Bradley, H. C., *Physiol. Rev.*, **2**, 415 (1922).
4. Hertzman, A. B., and Bradley, H. C., *J. Biol. Chem.*, **61**, 275 (1924).
5. Waldschmidt-Leitz, E., Beck, I. J., and Kahn, J., *Naturwissenschaften*, **17**, 85 (1929).
6. Bradley, H. C., and Morse, M., *J. Biol. Chem.*, **21**, 209 (1915).
7. Bradley, H. C., *J. Biol. Chem.*, **52**, 467 (1922).
8. Anson, M. L., *J. Gen. Physiol.*, **20**, 565 (1937).
9. Willstätter, R., and Baman, E., *Z. physiol. Chem.*, **180**, 127 (1929).
10. Waldschmidt-Leitz, E., and Schaffner, A., *Z. physiol. Chem.*, **188**, 17 (1930).
11. Kleinmann, H., and Stern, K. G., *Biochem. Z.*, **222**, 84 (1930).
12. Willstätter, R., and Rohdewald, M., *Z. physiol. Chem.*, **208**, 258 (1932).
13. Maschmann, E., and Helmert, E., *Z. physiol. Chem.*, **216**, 141 (1933).

VITAMIN A AND LIVER CELL TUMORS

By ALFRED GOERNER and M. MARGARET GOERNER

(From the Department of Biological Chemistry, Long Island College of Medicine, and the Department of Pathology, Brooklyn Thoracic Hospital, Brooklyn, New York)

(Received for publication, March 4, 1939)

The importance of vitamin A for normal growth has led to its use by some workers (1) in an attempt to influence the growth of neoplasms. The present authors (2) have shown, however, that transplants of Flexner-Jobling rat carcinoma and R-39 rat sarcoma contain no detectable amounts of vitamin A, even though large amounts of it were found stored in the hepatic tissues of the hosts. Since the transplants were not situated close to the liver and because the tumors themselves did not originate in that organ, it was decided to study the vitamin A content of primary tumors developing in tissues naturally rich in the vitamin.

The experiments of Yoshida (3), the results of which are also to be found in a report by Sasaki and Yoshida (4), demonstrated that 2-amino-5-azotoluene (*o*-amidoazotoluene) regularly produces hepatomas in rats. Zylbersac (5) was able to confirm these results and the work of Nishiyama (6) and Shear (7) gave further confirmation of the carcinogenic action of the dye in mice. Such liver cell tumors seemed ideal for the work that was contemplated and the compound was therefore used in the following experiments.

EXPERIMENTAL

A group of 130 white rats each weighing from 30 to 50 gm. was fed the ordinary commercial chow for laboratory animals to which had been added 0.1 per cent 2-amino-5-azotoluene. A control group of forty rats of approximately the same age and weight was kept under similar conditions and on the same diet except for the dye. Little difference was noticed between the two groups of animals except for the yellowish stains that dis-colored the fur of the treated rats. All of them continued to gain weight, however, and appeared to suffer from no toxic effects of the dye. After 4 weeks the concentration of the dye was increased to 0.2 per cent, which is slightly greater than the largest concentration used by Yoshida (3). Since this proved to be somewhat more toxic than the former concentration, it was reduced again to 0.1 per cent at the end of 2 weeks. Not much change in the external appearance of the animals was noticed until 7 months had passed, when those of the treated group became unkempt and dull and showed a loss of hair. This became more marked during the following months. Its explanation probably lies in the great burden placed on the detoxicating mechanism by the continued absorption of the dye, for if such animals were given food containing no dye improvement quickly resulted.

At intervals during the period that preceded the appearance of hepatic tumors two animals from each group were sacrificed by exsanguination, after which a careful examination was made of the thoracic and abdominal organs. The one constant finding of this pretumorous period was a very noticeable enlargement of the liver in the treated animals. The control rats were normal in all instances. After the liver had been weighed, the estimation of its vitamin A content was immediately begun by extracting it according to Moore's method (8). The residue obtained on evaporating the ether extract was dissolved in chloroform and the vitamin A determined colorimetrically by both the method described by Rosenthal and Erdélyi (9) and a modification proposed by Andersen and Levine (10). Both of these methods are based on the reaction that vitamin A gives with a saturated solution of antimony trichloride in chloroform. A biologically standardized preparation of the vitamin suitably diluted with chloroform was used for comparison. Some of the results of the pretumorous period up to and including the 40th week are shown in Table I.

In two of the treated rats that died in the 28th and 32nd week showing marked abdominal distention autopsy revealed marked thickening and some sacculation of the proximal portion of the large bowel. Histologic examination showed necrosis of the mucous membrane and a granulomatous lesion infiltrating the submucosa and muscularis. There was considerable hyperplasia of lymphoid elements but no evidence of neoplasm.

TABLE I

Vitamin A Content of Liver Tissues and Hepatomas

Experiment No.	Stage of experiment	Vitamin A per 100 gm. liver tissue, U.S.P. units	Vitamin A per 100 gm. tumor tissue, U.S.P. units	Remarks
	wks.			
D3	20	5,130		Treated; liver enlarged
D6	20	4,050		“ “ “
C4	20	8,455		Control
C10	20	6,280		“
D12	33	3,209		Treated; liver 13.1 gm.
D15	33	1,560		“ “ 15.3 “
C7	33	9,115		Control
C16	33	12,411		“
D14	40	3,150		Treated; liver 18.5 gm.
D20	40	2,126		“ “ 21.4 “
C42	40	7,514		Control
C35	40	10,205		“
D48	55	1,425	0	Hepatoma
D101	55	5,064	0	“ rat on control diet for last 4 wks.
C28	55	8,460		Control
C39	55	11,236		“
D63	58	2,055	0	Hepatoma
D120	58	7,544	0	“ rat on control diet for last 7 wks.
C32	58	10,938		Control
C45	58	9,214		“
D87	60	3,240	0	Hepatoma
D62	60	9,847	0	“ rat on control diet for last 9 wks.
C36	60	7,512		Control
C44	60	11,965		“
D24	61	2,738	0	Hepatoma
D51	61	12,175	0	“ rat on control diet for last 10 wks.
C38	61	10,114		Control
C24	61	13,251		“
D105	58	10,880	604*	
D78	59	9,165	752*	
D91	61	8,744	490*	

In experiments marked D the animal was fed 2-amino-5-azotoluene added to commercial animal chow; in those marked C the animal was fed the same chow but containing no dye.

* Results obtained on analysis of entire tumorous lobe including liver tissue as well as hepatoma, for comparison with non-tumorous lobe (values in preceding column).

In the 55th week after the beginning of the experiments the first hepatic tumors were noticed in two rats of the treated group. During the 2 following months the remaining treated and control animals were sacrificed, the last one reaching autopsy 15 months after the start of the experiment. All of the twenty-eight treated rats that were examined subsequent to the first appearance of liver tumors in the 55th week showed hepatomas, the liver being studded at times with nodules. Enlargement of the organ was always found as against the normal size of the livers in the control group.

The liver was removed as quickly as possible and the tumors dissected from the surrounding liver tissues. Portions were reserved for histologic examination and the remainder carefully freed from any liver tissue that might be found adherent to the tumor. Estimation of the vitamin A content was begun immediately. The surrounding non-tumorous liver was separately analyzed for the vitamin.

Examination of the other organs was then made for possible metastases or other pathology. In three instances there were metastases to the lungs; except for these and the hepatomas no others tumors were found. The external appearance of the hepatic tumors was smooth and the consistency was practically that of normal liver tissue. Hemorrhages were noticed in some; none was found adherent to any of the surrounding organs.

On microscopic examination the tumors presented wide variations in structure not only as compared with one another but even in different portions of the same tumor. In some instances the change involved only a disturbance in the size and axis of the liver cords producing tortuous, large celled strands. At the other extreme liver structure was scarcely recognizable, many large acidophilic, multinucleated cells in disorderly arrangement making up the tumor nodule. Some of these showed areas of necrosis, others were hemorrhagic. Definite invasion of the blood vessels was demonstrable in some instances. In the case of two tumors the structure was that of adenocarcinoma.

Fragments of the hepatomas were transplanted subcutaneously into new hosts; in three cases the tumor was carried on for four generations of transplants. None of these showed vitamin A on analysis.

In order to give the liver every opportunity to store vitamin A, ten of the surviving twenty-eight rats were placed on the control diet which was free from dye. As this was done after 51 weeks of feeding the dye-containing diet, it could have had little effect on the development of the hepatomas, for Sasaki and Yoshida (3) found that tumor formation continues to go on in the liver, even if the ingestion of the dye was discontinued after an initial feeding of about 4 months. The appearance of the ten rats improved temporarily after they were placed on the control diet but this was soon offset by the effects of the growing hepatic tumors which were found later on autopsy.

All of the hepatomas showed an absence of vitamin A, both in those animals which were kept on the dye-containing diet throughout and in the rats that were placed on the control diet later in the experiment. The non-tumorous liver tissue of the latter animals showed, however, a much greater vitamin A content than the former, and in fact was comparable to the liver of the control rats in this respect. The vitamin A content of the neighboring liver tissues varied a great deal in those animals that were kept on the dye throughout the experiment. In one of these only 715 U.S.P. units per 100 gm. of liver tissue were found. Some of the results obtained on testing the hepatomas and the surrounding liver tissues of animals which had received the dye continuously and of those which had been given it for 51 weeks are shown in Table I. The tumor nodules subjected to analysis were selected for their freedom from hemorrhage or necrosis. The importance of this is evident.

In each of three animals one of the liver lobes was studded with tumor nodules while another lobe was found to be practically free. Advantage was taken of this to analyze the entire tumorous lobe including liver tissue and compare it with the non-tumorous lobe. The results showed that when the lobe was mostly made up of tumor tissue the vitamin A content was markedly reduced, being about one-tenth to nearly one-twentieth that of the non-tumorous lobe (Table I). All three tumorous lobes were free from necrotic changes.

Comments

The discovery of carcinogenic compounds of known chemical composition has given rise to much interesting work on the biological and chemical changes brought about by their presence in the tissues. The origin of the present study goes back to experiments on the effect of such a carcinogen, 1,2,5,6-dibenzanthracene, on hepatic mitochondria (11). One of the results observed at that time was the rapid depletion of vitamin A brought about by the hydrocarbon in these cytoplasmic structures. Experiments were then started to correlate this finding with the results of Haddow, Scott, and Scott (12) and Lees (13) on the growth-inhibiting action of dibenzanthracene in young animals. It was found that the sudden and prolonged retardation of growth was parallelled by the rapid and continued absence of vitamin A from the hepatic tissues.[1] The importance of vitamin A in growth processes made its study in rapidly growing tumors a logical sequence Analysis of Flexner-Jobling rat carcinoma and R-39 rat sarcoma showed complete absence of vitamin A although the hepatic tissues contained large amounts of it (2).

Because of this finding it was decided to study the effect of a carcinogenic compound which produces tumors of the liver parenchyma itself. It was found that 2-amino-5-azotoluene reduces the vitamin A content of the hepatic tissues and produces hepatomas which show no detectable vitamin A even though the surrounding liver tissue may contain large amounts of it.

It would seem from these results that some cells which are able to survive the vitamin A-depleting action of a carcinogen develop a growth pattern in which vitamin A is not an integral part.

SUMMARY

1. Rats were fed a diet containing 0.1 per cent 2-amino-5-azotoluene for 1 year.

2. The feeding of the dye caused a reduction of vitamin A content in the hepatic tissues.

3. Analysis of the liver cell tumors which developed after prolonged feeding of the dye showed an absence of vitamin A.

4. The hepatic tissues surrounding the hepatomas were able to store vitamin A.

BIBLIOGRAPHY

1. Sure, B., Buchanan, K. S., and Thatcher, H. S., *Am. J. Cancer*, **27**, 84 (1936).
2. Goerner, A., and Goerner, M. M., *J. Biol. Chem.*, **123**, 57 (1938).

---

[1] Goerner, A., unpublished data.

3. Yoshida, T., *Tr. Japan. Path. Soc.*, **24**, 523 (1934).
4. Sasaki, T., and Yoshida, T., *Virchows Arch. path. Anat.*, **295**, 175 (1935).
5. Zylbersac, S., *Compt. rend. Soc. biol.*, **125**, 389 (1937).
6. Nishiyama, Y., *Gann*, **29**, 285 (1935).
7. Shear, M. J., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **114**, p. xc (1936).
8. Moore, T., *Biochem. J.*, **24**, 696 (1930).
9. Rosenthal, E., and Erdélyi, J., *Biochem. Z.*, **267**, 119 (1933).
10. Andersen, A. C., and Levine, V. E., *Proc. Soc. Exp. Biol. and Med.*, **32**, 737 (1935).
11. Goerner, A., *J. Biol. Chem.*, **122**, 529 (1937–38).
12. Haddow, A., Scott, C. M., and Scott, J. D., *Proc. Roy. Soc. London, Series B*, **122**, 477 (1937).
13. Lees, J. C., *Quart. J. Exp. Physiol.*, **27**, 161 (1937).

DIURNAL VARIATIONS OF HEMOGLOBIN IN THE BLOOD OF NORMAL MEN

By EARL F. McCARTHY and DONALD D. VAN SLYKE

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, March 11, 1939)

The occurrence of diurnal variations of hemoglobin in the blood of normal people has been reported by a number of observers. Dreyer, Bazett, and Pierce (1) and Rabinowitch (2) have made determinations of the hemoglobin content of the blood of normal adults during the day and find a possible variation of 20 to 30 per cent of the average content. Short (3) finds a possible variation of 17 per cent as a result of his experiments. The methods employed by these investigators have not been so precise as those now available. The colorimetric methods used by Dreyer and his coworkers and by Short do not allow for the effect of variations in substances other than hemoglobin, such as lipids, which could affect the light absorption of the samples. Rabinowitch used the oxygen capacity method in his experiments, but employed an earlier and considerably less exact technique; also the inhalation of small amounts of CO by smokers may cause an appreciable alteration of the apparent hemoglobin content. His maximum variation during the day was more than double that observed by us.

Methods

The subjects in these experiments were healthy men from 20 to 30 years of age. They comprised laboratory technicians and medical students who were performing ordinary routine work. Blood was obtained by venipuncture from the arm veins at 2 to 3 hour intervals throughout the day. Precautions were taken to avoid stasis. The hemoglobin content was estimated from the CO-combining capacity according to the method of Van

Slyke and Hiller (4). In this technique the blood sample mixed with water is saturated with CO in the 50 cc. chamber of the Van Slyke manometric apparatus. Subsequently the excess CO and the O_2 and N_2 extracted by the shaking are expelled from the chamber, and the HbCO is determined by measurement of the CO set free by acid ferricyanide solution.

We used samples of 1 cc. of blood, and measured the pressure of the CO gas at 1 cc. volume. (A Van Slyke-Neill chamber calibrated at 1.0 cc., in addition to the usual 0.5 and 2.0 cc. points, was used.) These conditions gave a desirable combination of accuracy with economy of blood. The mean difference between duplicate analyses was 0.06 volume per cent of CO capacity.

The CO capacity method has over the O_2 capacity two advantages with respect to immunity from error.

1. The correction for physically dissolved O_2 is replaced by a smaller and practically constant correction for physically dissolved CO. According to the solubility determinations of Sendroy, Dillon, and Van Slyke (5) normal blood saturated with air at 20° under 760 mm. of atmospheric pressure dissolves 0.70 per cent of O_2, and in blood of abnormal cell content the dissolved O_2 varies with the cell content. In comparison the correction for CO dissolved in the blood plus 2.5 volumes of water is only about 0.33 volume per cent of the blood in the technique of Van Slyke and Hiller, and is relatively independent of the cell content.

2. If the blood contains any preformed CO, as from tobacco smoke or the exhaust of automobiles, the O_2 capacity values are lowered, while the CO capacity is unaffected. The reason for this is that the CO is so strongly bound to the hemoglobin that agitation with air for the periods ordinarily used fails to replace the HbCO entirely with HbO_2. The O_2 subsequently determined in such a case therefore does not indicate all the Hb present. That the amount of CO in the blood of modern man can suffice to lower significantly the O_2 capacity values is indicated by the data on p. 112 of Enghoff's (6) monograph. He found O_2 capacities significantly lower than CO capacities in the bloods of seventeen out of 168 subjects; in these seventeen subjects the CO capacity exceeded the O_2 capacity by from 0.4 to 1.7 volume per cent.

TABLE I

Hemoglobin Concentrations of Normal Young Men at Different Hours of the Day

Subject	Cc.CO capacity for 100 cc. blood									Range of day in per cent of median
	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	11 p.m.	Average for subject	Median for day	Range of day above and below median	
W. D.	20.63	20.99	20.88	20.79	19.68	19.19	20.39	20.09	±0.90	±4.5
G. M.	20.03	19.00	19.26	19.24	18.93	18.46	19.16	19.25	±0.79	±4.1
F. W.	20.32	20.02	19.92	19.61	18.95	19.52	19.73	19.62	±0.70	±3.6
J. K.	18.84	19.97	18.82	19.27	18.12	18.45	18.92	19.05	±0.92	±4.8
F. R.	19.62	19.73	20.13	20.19	19.30	20.75	19.97	20.03	±0.72	±3.6
H. G. T.*	17.21	16.67	17.16	17.01	17.08	16.70	16.99*	16.94*	±0.27	±1.6
F. B.	19.88	19.45	19.90	18.89	19.21	18.86	19.38	19.90	±0.55	±2.8
J. F.	20.77	20.88	20.47	21.37	20.36	20.31	20.70	20.84	±0.53	±2.5
J. M.	20.56	20.72	20.59	19.92	19.86		20.33	20.29	±0.43	±2.1
J. B.	21.13	20.54	21.13	20.07	21.02	20.19	20.68	21.13	±0.53	±2.5
A. C.	21.63	20.33	19.36	19.52	19.59	19.31	19.95	20.50	±1.14	±5.4
J. S.	20.16	20.35	20.04	19.53	19.02	19.22	19.72	19.69	±0.67	±3.4
F. P.	19.38	18.93	18.83	19.14	18.87	18.61	18.96	19.00	±0.39	±2.0
R. S.	18.59		17.77	17.03	17.63	17.46		17.81	±0.78	±4.4
	18.65	18.23	17.93	18.26	18.63	18.24	18.02	18.29	±0.36	±2.0
D. K.	20.57	20.04	20.03	20.30	20.34	20.62		20.33	±0.30	±1.5
	21.30	21.06	20.55	20.39	20.31	20.04	20.48	20.67	±0.63	±3.1
E. J.	20.67		19.61	19.67	19.43	19.69		20.05	±0.62	±3.1
	20.62	20.07	19.38	19.33	19.25	18.99	19.70	19.81	±0.82	±4.1
R. E.	20.24	21.33	20.86	21.06	21.70	21.17		20.97	±0.73	±3.5
	21.48	21.92	21.31	21.41	21.20	20.70	21.20	21.31	±0.61	±2.9
L. A.	20.78	21.35	19.95	20.81	20.15	19.62		20.49	±0.87	±4.2
	19.87	20.00	19.63	19.52	19.34	19.01	20.02	19.51	±0.50	±2.6
Maximum							21.20	21.31	±1.14	±5.4
Minimum							18.02	17.81	±0.27	±1.6
Mean							19.86	19.94	±0.64	±3.2
Standard deviation $\sqrt{\Sigma d^2/n}$							±0.74	±0.92	±0.21	±1.0

* The exceptionally low data for H. G. T. are excluded in calculating means, minima, and standard deviations for "average for subject" and "median for day."

Results

The results from eighteen subjects are shown in Table I. In thirteen of the subjects the observations covered 1 day each; in five subjects observations were made on 2 separate days about a week apart. Fig. 1 illustrates the changes observed in the five subjects who provided two curves each. From Fig. 1 there seems to be a tendency for the blood of each subject to follow a daily course typical of the individual.

The greatest range between the highest and lowest values for 1 day was 2.3 volumes per cent of CO capacity, or 11 per cent of the mean value. The average range was 1.3 volumes per cent of CO capacity, or 6.4 per cent of the mean.

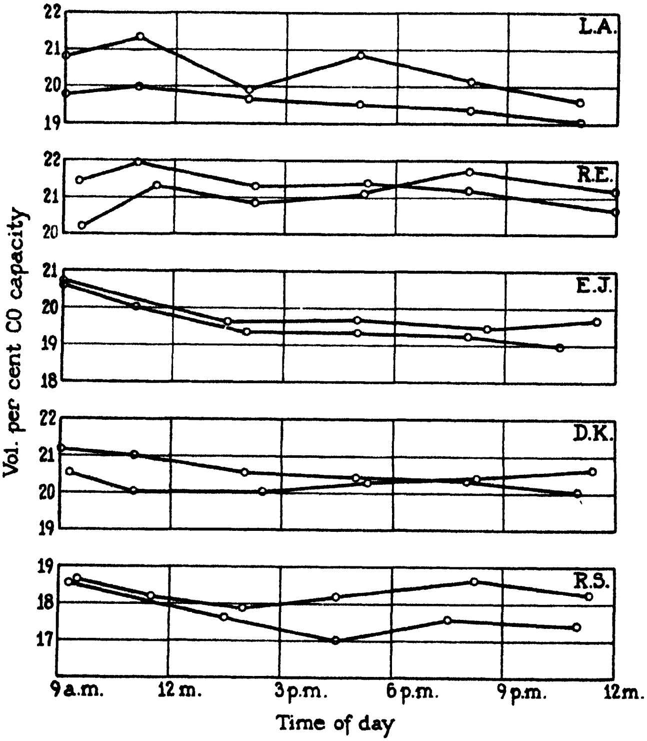


FIG. 1. Diurnal hemoglobin variations observed on 2 different days, about 1 week apart, in five young men.

These ranges of variation are narrower than those reported with earlier, and probably less exact, methods for hemoglobin measurement.

The direction of change in hemoglobin concentration during the day is not predictable from our data. There seems to be a tendency for the concentration to decrease during the waking hours: in 20 of the 23 days observed the hemoglobin at 11 in the evening was less than at 9 in the morning. The variation in behavior is such, however, that on a given day one could not predict even the direction of the change. Thus subject R. E. showed, from the earliest to the latest blood sample on 1 day, a decrease from 21.48 to 20.70, and on another day an increase from 20.24 to 21.17 volumes per cent.

The mean hemoglobin content of our eighteen subjects is represented by 19.9 volumes per cent of CO capacity. It appears that most of our subjects were in the lower half of the range, 20.7 ± about 2.0, for normal men, which was estimated from data available in 1930 by Peters and Van Slyke (7), and has been confirmed recently by Enghoff's (6) precise study, with the CO capacity method applied to 95 men between 18 and 59 years of age.

SUMMARY

In eighteen young men hemoglobin determinations by Van Slyke and Hiller's manometric CO capacity method were performed at intervals of 2 to 3 hours from 9 a.m. to 11 p.m. on 1 or more days. The accuracy is indicated by a mean difference between duplicate analyses of 0.06 volume per cent of CO capacity.

The range between highest and lowest values in an individual through a day averaged 1.3 volumes per cent; the greatest range seen in any of the 23 observed days was 2.3 volumes per cent, equivalent to 11 per cent of the mean hemoglobin content for the day. Usually the hemoglobin content was lower in the evening than in the morning; but a subject showing this change on one day reversed it on another.

The much greater ranges of diurnal hemoglobin variation reported in the past literature appear attributable to methods of analysis which provided a significant part of the variations.

BIBLIOGRAPHY

1. Dreyer, G., Bazett, H. C., and Pierce, H. F., *Lancet*, **2**, 588 (1920).
2. Rabinowitch, I. M., *J. Lab. and Clin. Med.*, **9**, 120 (1923–24).
3. Short, J. J., *J. Lab. and Clin. Med.*, **20**, 708 (1934–35).

4. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, **78**, 807 (1928). Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 341 (1932).
5. Sendroy, J., Jr., Dillon, R. T., and Van Slyke, D. D., *J. Biol. Chem.*, **105**, 597 (1934).
6. Enghoff, H., Quantitative Hämoglobinbestimmungen und Erhthrozytenuntersuchungen, Upsala (1937).
7. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Interpretations, Baltimore, 544 (1931).

THE DETERMINATION OF THE UREA IN CHICKEN BLOOD

By STACEY F. HOWELL

(*From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca*)

(Received for publication, February 3, 1939)

The observation was made some 6 years ago that chickens were not poisoned by the parenteral injection of large quantities of crystalline urease, unless urea was also injected (1). However, rabbits were killed after urease was injected when the blood ammonia nitrogen reached a value of 5 mg. per 100 cc. (2). These observations offer sufficient evidence to make many of the values reported for urea nitrogen in chicken blood, shown in Table I, questionable. Analyses of chicken blood, when the urea is hydrolyzed with crystalline urease, showed that the value for urea nitrogen was much lower than had been reported. According to Table I, there has been a gradual decrease in the values recorded for urea nitrogen in normal chicken blood during recent years until the average value of 0.7 mg. per 100 cc. of blood, as reported in Table II, is almost reached. The largest quantity of urea nitrogen found in the blood of forty-five normal chickens was 2.1 mg. per 100 cc. and the lowest 0.0 mg.

Since the values obtained for the urea nitrogen content of normal chicken blood are extremely low, errors in methods for the determination of blood urea can be detected by the use of chicken blood. While the use of chicken blood will not disclose errors of less than 2 mg. of urea nitrogen per 100 cc. with any given method, it will enable the investigator to avoid many of the larger errors which have appeared in publications.

The correct determination of the quantity of urea nitrogen in the blood of normal chickens is of value in the study of changes caused by disease. It has been reported that there is no change in the values for blood urea nitrogen of chickens with Rous sar-

TABLE I

Values Reported for Urea Nitrogen Content of Normal Chicken Blood

Authors	Urea N, mg. per 100 cc. blood	Publication	Method used
Folin, O., and Denis, W.	8.0	*J. Biol. Chem.*, **14**, 29 (1913)	Heat hydrolysis
Karr, W. G., and Lewis, H. B.	6–12	*J. Am. Chem. Soc.*, **38**, 1615 (1916)	Urease method; Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, **18**, 53 (1914)
Scheunert, A., and von Pelchrizim, H.	12.1	*Biochem. Z.*, **139**, 17 (1923)	Urease method; Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919)
Pupilli, G.	9.1	*Arch. ital. biol.*, **73**, 181 (1924)	Salkowski method; Arbeiten aus dem pathologischen Institut, Berlin, 581 (1906)
Hayden, C. E., and Fish, P. A.	5.4	*Cornell Vet.*, **18**, 197 (1928)	Urease method; Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919)
Hayden, C. E., and Sampson, J.	Trace to 4.5	*Rep. New York State Vet. Coll.*, 174 (1929–30)	“ “
Howell, S. F.	1.0 or less	*Proc. Soc. Exp. Biol. and Med.*, **29**, 759 (1932)	Crystalline urease, aeration and titration
Dyer, H. M., and Roe, J. H.	3.3	*J. Nutrition*, **7**, 623 (1934)	Pressure hydrolysis; Leiboff, S. L., and Kahn, B. S., *J. Biol. Chem.*, **83**, 347 (1929)
Gonzaga, A. C.	1.2–2.6	*Rep. New York State Vet. Coll.*, 53 (1933–34)	Urease, direct nesslerization; Karr, W. G., *J. Lab. and Clin. Med.*, **9**, 329 (1924)
Eveleth, D. F.*		*J. Lab. and Clin. Med.*, **19**, 783 (1934)	Nesslerization of urease-treated blood filtrate

Heller, V. G., and Pursell, L.	3.6	*J. Biol. Chem.*, **118**, 549 (1937)	Urease-heat distillation; Folin, O., and Svedberg, A., *J. Biol. Chem.*, **88**, 77 (1930)
Shimer, S. R.	7.2	*New Hampshire Agric. Exp. Stat., Tech. Bull. 69* (1937)	Karr, W. G., *J. Lab. and Clin. Med.*, **9**, 329 (1924). Folin, O., and Svedberg, A., *J. Biol. Chem.*, **88**, 77 (1930)
Pitts, R. F., and Korr, I. M.	0.0–1.7, serum used	*J. Cell. and Comp. Physiol.*, **11**, 117 (1938)	Manometric urease; Van Slyke, D. D., *J. Biol. Chem.*, **73**, 695 (1927)

* "Fowl blood does not lend itself to the determination of urea by this method."

coma (3) or with range paralysis (4), since the values obtained with the bloods of normal and diseased chickens were almost the same. However, the two normal values reported are 3.3 and 7.2 mg. of urea nitrogen per 100 cc. as compared with the value of 0.7 mg. reported above. While there may be some variation in normal values, this variation is excessive, and is probably due to the erroneous methods used.

Since normal chicken blood has been found to contain very little urea, it was of interest to determine whether chicken blood contained measurable quantities of urea under pathological conditions. Definitely increased quantities were found in the blood of seventeen of the forty-three diseased hens investigated. The blood of three of these diseased hens contained between 7.0 and 8.0 mg. of urea nitrogen. According to the values recorded in Table III, the average urea nitrogen content of the blood of the diseased hens analyzed is 1.9 mg. per 100 cc.

TABLE II

Urea Nitrogen Content of Blood of Normal Chickens

Breed	Sex	Age	No. of chickens	Average values, mg. N in 100 cc. blood		
				Urea + ammonia N	Ammonia N	Urea N
White Leghorn	Male	8 wks.	20	1.0	0.6	0.4
" "	Hens, laying	1 yr.	7	1.7	1.0	0.7
Plymouth Rock	Male	8 wks.	4	1.5	1.0	0.5
Rhode Island Red	"	8 "	2	2.0	0.8	1.2
Cross-breed	"	1 yr.	10	2.2	1.0	1.2
"	Hen	1 "	1	1.4	0.8	0.6
Average			45	1.5	0.8	0.7

The cause of the higher urea nitrogen values found in a number of the diseased hens has not been determined. As the higher values were not found consistently in any one disease, they may have been due to a greater degree of development of the disease.

The method used in the determination of urea nitrogen in chicken blood will be published later. Crystalline urease was used to hydrolyze the urea in 5 cc. of blood, since jack bean meal often contains sufficient preformed ammonia to cause an appreciable error when the quantity of urea nitrogen is below 1.0 mg. per 100 cc. of blood. In a few instances the presence of urea in the blood of diseased chickens was verified by the formation of a precipitate when xanthydrol was added to the blood filtrate according to the procedure of Beattie (5).

TABLE III

Urea Nitrogen Content of Blood of Diseased Hens

The nitrogen values are expressed in mg. per 100 cc. of blood.

Disease	Age	Breed	No. of hens	Urea + ammonia N		Ammonia N		Urea N	
				Average	Extremes	Average	Extremes	Average	Extremes
Range paralysis	3 mo.–1 yr.	White Leghorn	22	2.6	0.5–9.4	0.7	0.3–1.9	2.0	0.0–7.9
Tumors	1 yr.	“ “	4	3.0	0.9–5.4	0.8	0.7–0.9	2.2	0.2–4.5
Tapeworms and coccidiosis	3 mo.–1 yr.	“ “	6	2.3	0.7–4.7	0.8	0.5–1.2	1.5	0.2–3.8
		New Hampshire Red	1						
Respiratory disease	1 yr.	Rhode Island Red	3	2.8	0.6–7.8	0.4	0.2–0.7	1.4	0.4–7.5
		White Leghorn	1						
Egg sac disturbance	1 “	White Rock	2	1.8	0.9–2.9	0.4	0.1–0.6	1.4	0.5–2.8
		“ Leghorn	1						
Miscellaneous	3 mo.–1 yr.	“ “	3	1.9	0.9–3.2	0.7	0.5–0.9	1.2	0.2–2.7
Average			43	2.56		0.66		1.9	

The ammonia nitrogen content of normal and diseased chickens recorded in Tables II and III was determined to obtain the corrected values for urea nitrogen.[1] The ammonia nitrogen values include the preformed ammonia as well as the ammonia formed during the 1 hour aeration of the blood in the presence of powdered potassium carbonate and are probably too high. There was no definite relationship between the ammonia nitrogen and urea nitrogen values.

---

[1] The samples of blood from normal chickens were collected when the chickens were slaughtered, by Mr. F. E. Andrews of the Poultry Department of Cornell University. The chickens were fed a stock diet and had fasted 24 hours. The blood of diseased hens was obtained from the Poultry Disease Laboratory of the New York State Veterinary College. The hens had been received from poultrymen living in various parts of the state of New York.

SUMMARY

The values published for the urea nitrogen content of normal chicken blood are generally too high, owing to erroneous methods used in the analyses. The average value found is 0.7 mg. per 100 cc. The urea nitrogen content of chicken blood may increase owing to disease.

BIBLIOGRAPHY

1. Howell, S. F., *Proc. Soc. Exp. Biol. and Med.*, **29**, 759 (1932).
2. Kirk, J. S., and Sumner, J. B., *J. Biol. Chem.*, **94**, 21 (1931-32).
3. Dyer, H. M., and Roe, J. H., *Am. J. Cancer*, **18**, 888 (1932); *J. Nutrition*, **7**, 623 (1934).
4. Shimer, S. R., *New Hampshire Agric. Exp. Stat., Tech. Bull. 69* (1937).
5. Beattie, F., *Biochem. J.*, **22**, 711 (1928).

UNSTABLE ISOTOPES

I. THE DETERMINATION OF RADIOACTIVE ISOTOPES IN ORGANIC MATERIAL*

By ERWIN CHARGAFF

(From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, March 13, 1939)

In the course of experiments on the formation of radioactive phosphatides in the body and on the synthesis of radioactive organic substances containing the unstable isotope[1] P^{32}_{15} it was found of advantage to determine the radioactivity of the compounds themselves rather than of their ignition residues, as is commonly done (2). This procedure makes it possible subsequently to recover the radioactive substances unchanged and to submit them to further purification or other chemical operations.

For the expression of the radioactivity of the compounds tested and for the standardization of the various vessels employed a convenient way was found in the use of *potassium fluoride*. All potassium occurring in nature is known (3) to contain the unstable isotope K^{40}_{19} in a concentration estimated between 3×10^{-8} and 4×10^{-6} gm. per gm. The decay of the unstable potassium isotope is an extremely slow process; its half lifetime has been calculated (3) to lie between 7×10^{7} and 5×10^{10} years. Potassium fluoride was chosen because of its high solubility in water. The β-rays emitted by a given amount of potassium fluoride under comparable conditions form a convenient and easily reproducible standard. *The radioactivity of the preparation is expressed in KF units, which are defined as the amount of potassium fluoride in*

---

* This work has been supported by a grant from the John and Mary R. Markle Foundation.

[1] In writing formulas, analytical figures, etc., for compounds containing an unstable isotope an asterisk before the letter symbol for the particular element will be used (*e.g.* $Na_3{*}PO_4$), as proposed in a previous note (1).

mg. which under standard conditions shows the same degree of radioactivity as a given amount of the substance measured.

EXPERIMENTAL

Measurement of Radioactivity

The radioactivity of the substances was determined by means of a Geiger-Müller counter.[2] In order to minimize the effects of local γ radiation, cosmic rays, etc., the counter was enclosed in a chamber, a diagram of which is shown in Fig. 1, consisting of a heavy lead cylinder with walls 3 cm. thick, which could be used in both the horizontal and vertical positions. A medium sized counter tube made out of thin glass was employed. Its active surface was defined by the thin, cylindrical silver cathode E, which had a length of 5 cm. and a diameter of 1.6 cm. The

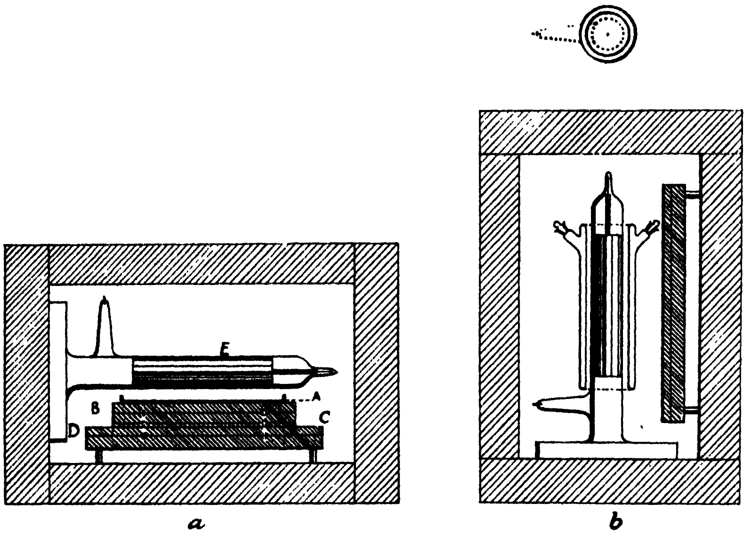


FIG. 1. Counting chamber for the measurement of radioactivity in organic substances (*a*) in the dry state and (*b*) in solution. *A* is an aluminum tray, *B*, *C*, and *D* are bakelite plates, and *E* is the silver cathode.

---

[2] We are highly indebted to Dr. J. R. Dunning and Mr. H. A. Glassford of the Department of Physics of this University for the construction of the apparatus.

silver was deposited chemically. The recording amplifier system had a high resolving power. Each impulse received was recorded in a loud-speaker and every second impulse was recorded on a mechanical counter by means of a suitable thyratron tube arrangement. The background effect of the counter corresponded to 12 impulses per minute.

Determination of Radioactivity in Organic Material

For the determination of radioactive isotopes in organic substances two methods were used: (1) The substance, in the dry state, was spread in a thin layer (thickness approximately 0.5 mm.) on a small aluminum tray which was placed underneath the counter tube at a standard distance. (2) The substance, dissolved in a suitable solvent, was placed in the hollow jacket of a double walled, thin glass cylinder which was slipped over the counter tube. The first arrangement is preferable in cases in which small amounts of material or substances of low radioactivity are to be tested.

Measurement in Dry State—The arrangement used is shown in Fig. 2.[3] The tray *A* has a trough 58 mm. long, 27 mm. wide, and 1 mm. deep, and is stamped out of an aluminum sheet 76 × 35 mm. By means of a simple interlocking device the tray is connected with the bakelite plates *B*, *C*, and *D*, which are 140 mm. long, 50 mm. wide, and 5 mm. high. In this manner a solid block is obtained which slides into a groove on a support underneath the counter tube, as shown in Fig. 1, *a*.

This arrangement makes it possible to carry out the measurements at one of three standard distances from the cathode, the distance being selected in accordance with the activity of the preparation. With all three plates *A*, *B*, and *C* in use the distance between the material measured and the counter tube is 2 mm. (Distance 1); with plates *B* and *C* the distance is 7 mm. (Distance 2); with plate *C* only the distance is 12 mm. (Distance 3). On the basis of a number of measurements with various radioactive substances the number of impulses registered when Distances 1, 2, and 3 were used was found to be in the ratio of 2:1.4:1.

Measurement in Solution—For determining the activity of solutions double walled, thin glass cylinders were used which in vertical position were slipped over the counter tube, as shown in Fig. 1, *b* in side and plan views. The cylinders were about 110 mm. high, and had an outside diameter of 30 mm. and an inside diameter of 22 mm. The width of the hollow jacket was 1.5 mm. The thickness of the glass walls varied between 1 and 1.5 mm. In all vessels used 10 cc. of solution were sufficient to cover the entire area surrounding the cylindrical silver cathode.

---

[3] We should like to thank Mr. F. Rosebury of this Department for its construction.

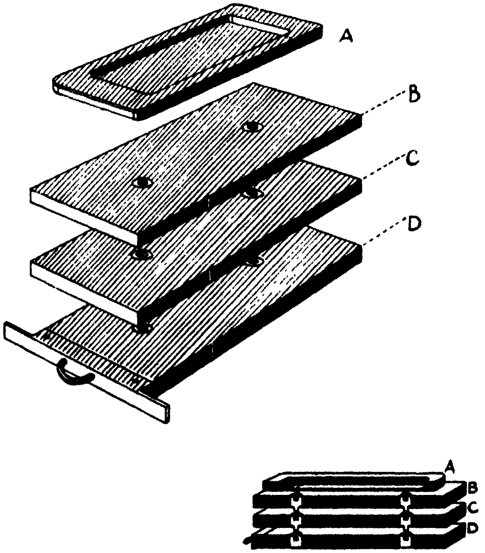


Fig. 2. Arrangement for the measurement of radioactivity in the dry state. *A* is an aluminum tray and *B*, *C*, and *D* are bakelite plates.

For volatile solvents and measurements of long duration vessels with closed jackets were used which, as shown in Fig. 1, *b*, had at the upper end two short tubulations with ground-in glass stoppers through which the solutions could be admitted and withdrawn. For aqueous solutions vessels with jackets that were open at the top were found more convenient.

Because of varying thickness of the cylinder walls and of the jackets containing the solution the amount of β-rays transmitted from the same substance will not be identical for different vessels.

The use of potassium fluoride as a radioactive test substance, as described in the following paragraphs, makes it possible to reduce results obtained under different conditions to a standard value. The absolute values of the radioactivity of different radioactive isotopes will be comparable only if the energy spectra of the radiation emitted (electrons, positrons, and γ-rays) are the same. The relative values for any given material, however, are accurately comparable.

Potassium Fluoride As Standard

As the test substance potassium fluoride (Merck's reagent) was used, of which 50.0 gm. were dissolved in water to give a total volume of 100 cc. The solution was stored in a paraffin-coated bottle. In Table I measurements[4] are reproduced which were carried out with 10 cc. of a potassium fluoride solution containing 5.0 gm. of KF and with 10 cc. of a 0.34 per cent aqueous solution of $Na_3{}^*PO_4 \cdot 12H_2O$ which contained a very small amount of radioactive P. It will be seen that in the first series of determinations Vessel 2 was found to transmit 48.2 per cent and Vessel 3, 80.2 per cent of the β-rays transmitted by Vessel 1, whereas in the second series 47.8 and 80.6 per cent were found respectively.

TABLE I

Determination of Radioactivity in Glass Vessels

Vessel No.	Impulses per min.	
	10 cc. 50 per cent KF solution	10 cc. 0.34 per cent $Na_3{}^*PO_4 \cdot 12H_2O$ solution
1	62.0	103.9
2	29.9	49.6
3	49.8	83.7

The number of β-rays registered by the counter will be higher when the radioactive material is tested in the dry state than when it is in solution because in the latter case part of the radiation is absorbed by the solvent and the glass. In order to obtain comparable results it is necessary to find the relation between counts obtained in the dry state and those registered by a solution. This conversion factor can be easily arrived at with a radioactive preparation. With a preparation of $NaH_2{}^*PO_4 \cdot H_2O$ the following results were obtained. 1.2 mg. of the salt, spread on the aluminum tray at Distance 1, gave 187.3 impulses per minute. A solution of 6.8 mg. in 10 cc. of water, measured in glass Vessel 1, gave 168.9 impulses per minute. Therefore, under the conditions of the experiment 6.28 impulses obtained with the material in the dry state corresponded to 1 impulse registered by the same amount of substance in solution.

---

[4] All counts reported represent the averages of a number of measurements and are corrected for the background effect.

The activity of our radioactive preparations is expressed in *KF units; i.e.*, the amount of KF in mg. which, when measured in a glass cylinder in the form of a 50 per cent aqueous solution, shows the same degree of radioactivity as a given amount of the material tested under the same conditions. Let R be the radioactivity of a substance expressed in KF units per mg., A the number of impulses per minute registered by B mg. of this substance, and C the number of impulses per minute registered by D mg. of KF. We then have the equation

$$R = AD/BC \tag{1}$$

D/C is a constant which is characteristic for the measuring vessel. On the basis of the figures given in the second column of Table I D/C for Vessels 1, 2, and 3 is found to be 80.6, 167.2, and 100.4 respectively.

If the measurements are carried out in the dry state, a conversion factor f is applied which is characteristic for the distance between the counter tube and the material, and for the glass vessel with which comparison is made, as described above. With k as the vessel constant D/C, the equation will read

$$R = kA/fB \tag{2}$$

Examples—10 cc. of a solution containing a total of 34.0 mg. of $Na_3{}^*PO_4 \cdot 12H_2O$ were measured in Vessel 1 and found to give 103.9 impulses per minute. By application of Equation 1, 1 mg. of $Na_3{}^*PO_4 \cdot 12H_2O$ is found to contain 246 KF units.

1.2 mg. of $Na_2H{}^*PO_4 \cdot 12H_2O$, tested on the aluminum tray at Distance 1, gave 738.9 impulses per minute. By Equation 2 and with $f = 6.28$, as calculated above, 1 mg. of $Na_2H{}^*PO_4 \cdot 12H_2O$

is found to contain 7900 KF units. 2.2 mg. of the same preparation, dissolved in 10 cc. of water and measured in Vessel 1, gave 209.5 impulses per minute, corresponding to 7680 KF units per 1 mg. of the salt.

155.3 mg. of a radioactive lecithin preparation containing 3.6 per cent *P, tested on the aluminum tray at Distance 1, gave 28.3 impulses per minute. By Equation 2, 1 mg. of lecithin *P is found to contain 65 KF units.

It is a pleasure to acknowledge the assistance rendered by Mr. B. Kress in the course of these experiments.

SUMMARY

Methods for the determination of radioactive isotopes in organic compounds are discussed.

BIBLIOGRAPHY

1. Chargaff, E., *J. Am. Chem. Soc.*, **60**, 1700 (1938).
2. Chievitz, O., and Hevesy, G., *K. Danske Vidensk. Selsk., Biol. Medd.*, **13**, 9 (1937).
3. Hevesy, G., *Naturwissenschaften*, **23**, 583 (1935).

UNSTABLE ISOTOPES

II. THE RELATIVE SPEED OF FORMATION OF LECITHIN AND CEPHALIN IN THE BODY*

By ERWIN CHARGAFF

(*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, March 13, 1939)

In spite of a large amount of work concerning the seat of formation and the function of the phosphatides in the body, their actual place in the physiology of higher organisms is by no means fully established. It should, however, be noted that at least one important physiological function can be assigned to cephalin; *viz.*, that of being the prosthetic group in the specific protein, the thromboplastic factor, which is the natural activator of blood coagulation.

It has recently been pointed out (1) that the fact that lecithin and cephalin usually are obtained in conjunction by chemical procedures by no means connotes a common physiological function of the two. On the basis of the behavior of cephalin towards protamines it was concluded that it, unlike lecithin, could exist in the body in the form of a salt-like combination with certain proteins, and, therefore, could form part of the structural components of the organism.

In this connection it appeared of interest to investigate the relative speed of formation of lecithin and cephalin in the adult rat by means of the radioactive isotope P^{32}_{15} which has a half life-time of 14.5 days. It was hoped that such a study would throw light on the important question whether the mechanism of formation in the body of these two phosphatides was essentially similar.

The use of isotope labels in physiology, introduced by Hevesy in 1923 in a study of the absorption of lead by plants (2), has

---

* This work has been supported by a grant from the John and Mary R. Markle Foundation.

made it probable that even the depot substances of the organism show a much more rapid turnover than had been expected (3, 4), if the speed with which isotope labels appear in certain compounds actually is a measure of their turnover. It has to be noted that quantitative statements concerning newly formed compounds in different organs presuppose a common base-line with regard to the concentration of the isotope label, from which the synthesis starts. The question of synthesis in the organism, as followed by isotopes, will be discussed later in the paper.

EXPERIMENTAL

Preparation of Radioactive Sodium Phosphate

The radioactive phosphorus P^{32}_{15} used was prepared by the action of fast neutrons on carbon disulfide by a method essentially similar to that of Chievitz and Hevesy (5). 6 to 8 liters of purified CS_2 were irradiated with a radon-beryllium source for a period of 2 to 5 days. The solvent was distilled off in an all glass apparatus. The small amount of reddish residue was, in some cases after the addition of 30 mg. of dry red P, oxidized by repeated treatment with boiling concentrated HNO_3. The solution was concentrated, the acid was driven off by heating to 130°, and the temperature was raised to 150° until no acid vapors were given off. The residue was dissolved in water and brought by dilute NaOH to the pH corresponding to the primary, secondary, or tertiary salt; 50 to 100 mg. of inactive sodium phosphate were added, and the radioactive phosphate was obtained by evaporation of the water and drying. In these experiments $NaH_2{*}PO_4 \cdot H_2O$, $Na_2H{*}PO_4 \cdot 12H_2O$, and $Na_3{*}PO_4 \cdot 12H_2O$ were prepared.[1]

In cases in which very strong sources had been employed the phosphoric acid obtained was contaminated with a considerable amount of sulfuric acid, which had to be removed as $BaSO_4$ by addition of $BaCl_2$ to an acidified solution of the radioactive salt. The $BaSO_4$, even after prolonged washing with dilute HCl, still showed considerable radioactivity. From the filtrate $Ba_3({*}PO_4)_2$ was precipitated practically quantitatively by making the solution alkaline towards phenolphthalein with ammonia. The barium phosphate then was converted into $Na_3{}^*PO_4$ by means of the calculated amount of Na_2SO_4.

---

[1] In writing formulas, analytical figures, etc., for compounds containing an unstable isotope an asterisk before the letter symbol for the particular element will be used (*e.g.* $Na_3{*}PO_4$), as in the preceding paper (6).

The preparations used contained between 13,000 and 56,000 KF units per mg. of P, when measured by the method described in the preceding paper (6).

Measurement of Radioactivity

The radioactivity of the phosphatide fractions isolated was measured in the dry state as described previously (6).

The activity of the preparations is expressed in KF units per mg. of P. In a number of investigations dealing with the formation of radioactive phosphatides it seems to have been overlooked that the phosphorus values of body phosphatides, unless these are more rigorously purified than is possible with the small amounts available, may vary within wide limits. Since the radioactivity of a phosphatide is solely a function of the amount of *P present, the phosphorus content of each fraction must be determined, and the activity measurements must be correlated with it.

In order to take into account the natural decay of the unstable phosphorus and to express the activities of the preparations in terms of the radioactive phosphate fed, a standard measurement was taken with each set of measurements. The standard consisted in an intimate suspension of 0.5 to 1 mg. of the sodium phosphate fed in inert soy bean lecithin, the amount of which was chosen so as to approximate the weights of the phosphatides measured (compare (7)).

Separation of Phosphatides

The method chosen for the extraction and separation of the phosphatides had to permit a fairly rapid preparation of the material free of sphingomyelin and of inorganic phosphate. The quantitative recovery of the lipids was of minor importance.

The skinned carcasses and the individual organs of the rats were mechanically ground and dehydrated by treatment with acetone for 1 hour at room temperature. The acetone treatment was repeated with a fresh portion of the solvent, and the mixture was kept in the ice box for 12 hours. The dry material was then extracted at room temperature with a mixture of equal parts of freshly distilled absolute alcohol and ether for 16 to 24 hours, filtered off, and the extraction was repeated with hot alcohol-ether and finally with ether. The combined extracts were concentrated to dryness *in vacuo*, absolute alcohol was added to the residue, and the evaporation repeated twice. The residues then were dissolved in freshly distilled petroleum ether (boiling at 30–60°), between 2 and 10 gm. of finely powdered anhydrous Na_2HPO_4 were added, and the mixtures kept in the refrigerator overnight. This treatment effects the removal of small amounts of inorganic radioactive sodium phosphate, which might have been carried over into the extracts, by exchange with the large excess of sodium phosphate present (8). At the same time traces of *sphingomyelin* and *cerebrosides* are removed which are insoluble in cold petroleum ether. The filtrates were again treated with sodium phosphate, this time at room temperature for 2 hours.

The residues, after evaporation of the solvent, were taken up in a small amount of ether, centrifuged if necessary, and the *cephalin* precipitated with absolute alcohol. After chilling, the precipitates were centrifuged off, washed with chilled alcohol, and reprecipitated from their ethereal solution with alcohol. The cephalin fractions were finally washed with cold alcohol and acetone and dried *in vacuo*.

The alcohol-ether solutions containing the *lecithin* were evaporated to dryness and the residues dissolved in absolute alcohol and, if necessary, centrifuged. The solvent was removed, and the residue dissolved in ether, precipitated with acetone, and purified by reprecipitation.

All preparations were analyzed for phosphorus and, whenever possible, for nitrogen and amino nitrogen. In cases in which scarcity of material prevented the determination of amino nitrogen, the lecithin fractions were tested for the absence of free amino groups by means of the ninhydrin reaction (9).

Formation of Lecithin and Cephalin in the Body

In all experiments reported in this paper adult male rats weighing between 250 and 300 gm. were used, which were fed the ordinary laboratory diet. In one set of experiments on two rats each animal received 59.7 mg. of $NaH_2{}^*PO_4 \cdot H_2O$, dissolved in 1.2 cc. of water, by stomach tube; *i.e.*, a total of 13.4 mg. of $^*P$ with an initial activity of 175,000 KF units.

One animal was killed 19 hours, the other 43 hours after the ingestion of the radioactive sodium phosphate. The phosphatides were isolated in the manner described above. The results of the experiment are summarized in Table I. The values for the minimum amounts of newly formed phosphatides are obtained by comparing the activities of 1 mg. of *P fed with those of 1 mg. of lipid *P under identical conditions. In reality the amounts of newly formed lipids will be much larger because of dilution of the radioactive sodium phosphate fed with inactive phosphates present in the food and in the body.

TABLE I

Relative Speed of Formation of Lecithin and Cephalin in the Rat

Animal No.	Phosphatide	Weight	P	N	NH_2-N	Radioactivity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
		mg.	*per cent*	*per cent*	*per cent*	*KF units*	
1	Lecithin	1063.0	2.8	1.4		15.6	0.37
	Cephalin	353.4	3.0	1.4	1.2	17.6	0.42
2	Lecithin	935.3	3.1	1.5		23.7	0.52
	Cephalin	258.0	3.2	1.6	1.5	25.4	0.56

* The spread of the individual counts was within about 4 per cent of the mean values given.

Formation of Lecithin and Cephalin in Various Organs

Two rats of approximately 300 gm. body weight were used in another experiment in which each animal received 40.8 mg. of $Na_2H^*PO_4 \cdot 12H_2O$, dissolved in 2 cc. of water, by stomach tube; *i.e.*, a total of 3.53 mg. of *P with an initial activity of 198,000 KF units.

The animals were killed 24 hours after the ingestion of the sodium phosphate and the liver, brain, and intestinal tract were removed. The organs and the remaining carcasses were mechanically ground and extracted, as described above. The results of the experiment will be found in Table II. Since the amount of inorganic *P fed in this experiment was still smaller than in the preceding one, the figures in the last column of Table II can serve as an indication only of the relative apparent speeds of synthesis of the phosphatides in various organs. The absolute amounts of newly formed substances must have been considerably larger.

TABLE II

Relative Speed of Synthesis of Lecithin and Cephalin in Various Organs of the Rat

Animal No.	Organ	Phosphatide	Weight	P	Radioactivity* in 1 mg. *P	Minimum amount of newly formed phosphatide, in per cent of total phosphatide
			mg.	*per cent*	*KF units*	
3	Intestinal tract	Lecithin	172.2	2.2	61.6	0.30
		Cephalin	95.1	2.0	35.4	0.17
	Liver	Lecithin	109.0	3.2	81.3	0.37
		Cephalin	25.8	2.9	68.0	0.25
	Brain	Lecithin	45.6	3.0	19.7	0.09
		Cephalin	38.7	2.8	23.2	0.10
	Carcass	Lecithin	438.3	2.8	21.7	0.12
		Cephalin	337.0	2.1	22.1	0.12
4	Intestinal tract	Lecithin	86.3	2.4	57.6	0.28
		Cephalin	146.8	1.9	23.5	0.12
	Liver	Lecithin	212.4	2.9	69.6	0.32
		Cephalin	44.6	3.3	60.0	0.27
	Brain	Lecithin	56.2	3.2		
		Cephalin	36.8	3.0	18.6	0.08
	Carcass	Lecithin	448.0	3.0	15.4	0.09
		Cephalin	128.9	2.2	12.6	0.07

* The spread of the individual counts was within about 4 per cent of the mean values given.

DISCUSSION

The proof that the inorganic phosphorus of the food is partly converted into phosphatides in the organism, as demonstrated in studies of previous workers as well as in the present paper, hardly required the use of radioactive isotopes. It is, however, not possible to say at present whether the synthesis of phosphatides had taken place by exchange of the phosphoric acid group in the preformed molecule or by a new union of the structural elements of the compounds. The question of the stability of glycerophosphoric acid and of the phosphatides under various conditions will form the subject of a subsequent publication; it may, however, here be said that, with the accepted structures of lecithin and cephalin in mind, a mere exchange of the phosphoric acid group without breakage of its chemical linkages with the glyceride on one side and with the amino alcohol on the other appears highly unlikely.[2]

The experiments on the formation of total lecithin and cephalin in the body summarized in Table I seem to indicate that the synthesis of the two phosphatides proceeds at about the same speed. The study of the relative speeds of formation of lecithin and cephalin in various organs, however, reproduced in Table II, gave an entirely different picture. The intestinal tract was found to contain almost twice as much newly formed lecithin as cephalin, and the liver too contained significantly higher amounts of lecithin. That the comparison of the total amounts of lecithin and cephalin in the rat did not reveal this difference, is primarily due to the fact that the phosphatides of the eviscerated carcass constitute the major part of the phospholipids of the animal.

The finding that the phosphatides of the intestinal tract and of the liver show a considerably higher radioactivity than those of the brain and the rest of the organism is in harmony with similar results reported by Artom *et al.* (10) and later by Perlman *et al.* (7). (*Cf.* the review by Sinclair (11) on phosphatide metabolism.) This, as was pointed out in the introductory paragraphs, does not necessarily signify a higher degree of synthetic ability in these organs. A similar effect would be produced if the inorganic phosphorus were transported at different speed into the various organs where the phosphatide syntheses were taking place, or, for that matter, if the phosphatides themselves were migrating at unequal speeds into the different organs.

[2] "Synthesis of a compound," in the sense in which metabolic experiments with isotope labels can reveal it, will have to include all reactions by which an element is transferred to the compound in question. Whether such transfers can be effected by the living cell without the breakage of chemical links, cannot be decided at present.

The unexpected finding that the radioactivity of the lecithin from the intestinal tract and the liver was considerably higher than that of the cephalin seems to indicate that the syntheses of these phosphatides do not follow the same course.

The following reactions, if one disregards any less simple assumptions, could in the organism lead to the formation of phosphatides. (1) A diglyceride[3] is esterified with phosphoric acid to form a phosphatidic acid which in turn reacts with aminoethyl alcohol, choline, or a precursor of these alcohols. (2) Aminoethyl alcohol or choline is phosphorylated[4] and the resulting phosphoric acid esters combine with a diglyceride. (3) Lecithin is formed from cephalin (or one of its intermediates) by methylation of the free amino group (13, 14). The third assumption certainly is not borne out by the experiments reported here, which, as a matter of fact, would be in agreement with the opposite type of reaction, *viz.* the formation in the organism of cephalin from lecithin. Further work will be necessary to show by what reactions the phosphatides in the organism are built up.

The author is highly indebted to Dr. J. R. Dunning and associates of the Department of Physics of this University for help with the preparation of the radioactive phosphorus, to Dr. K. B. Olson of the Department of Surgery of this College for help with the animal experiments, and to Mr. B. Kress for general assistance.

SUMMARY

The relative speed of formation of lecithin and cephalin in the body of the rat and in various organs was followed by means of the radioactive phosphorus isotope. It was found that the synthesis of these phosphatides apparently did not take the same course, the intestinal tract and the liver being much richer in newly formed lecithin than cephalin. Some of the physiological aspects of these findings are discussed.

---

[3] The possibility that glycerophosphoric acid is formed first and the fatty acids are introduced afterwards is not considered here for reasons of brevity.

[4] The isolation of aminoethylphosphoric acid from tumor tissue has been reported by Outhouse (12).

BIBLIOGRAPHY

1. Chargaff, E., *J. Biol. Chem.*, **125**, 661 (1938).
2. Hevesy, G., *Biochem. J.*, **17**, 439 (1923).
3. Hevesy, G., *Enzymologia*, **5**, 138 (1938).
4. Schoenheimer, R., and Rittenberg, D., *Science*, **87**, 221 (1938).
5. Chievitz, O., and Hevesy, G., *K. Danske Vidensk. Selsk., Biol. Medd.*, **13**, 9 (1937).
6. Chargaff, E., *J. Biol. Chem.*, **128**, 579 (1939).
7. Perlman, I., Ruben, S., and Chaikoff, I. L., *J. Biol. Chem.*, **122**, 169 (1937–38).
8. Hahn, L., and Hevesy, G., *Skand. Arch. Physiol.*, **77**, 148 (1937).
9. Salisbury, L. F., and Anderson, R. J., *J. Biol. Chem.*, **112**, 541 (1935–36).
10. Artom, C., Sarzana, G., Perrier, C., Santangelo, M., and Segrè, E., *Arch. internat. physiol.*, **45**, 32 (1937). Artom, C., Sarzana, G., and Segrè, E., *Arch. internat. physiol.*, **47**, 245 (1938).
11. Sinclair, R. G., in Luck, J. M., Annual review of biochemistry, Stanford University, **6**, 245 (1937).
12. Outhouse, E. L., *Biochem. J.*, **30**, 197 (1936); **31**, 1459 (1937).
13. Trier, G., Ueber einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweissstoffe und Lecithine, Berlin, 47 (1912).
14. Witanowski, W. R., *Acta biol. exp.*, Warsaw, **2**, 61 (1928); *Ber. ges. Physiol. u. exp. Pharmakol.*, **49**, 602 (1929).

SOME REACTIONS OF AMMONOLYZED INSULIN

BY RICHARD G. ROBERTS

(From the Department of Physiological Chemistry, Chicago Medical School, Chicago)

(Received for publication, February 27, 1939)

In previous papers (1, 2) it was reported that proteins are acidic in liquid ammonia and liberate hydrogen when sodium is added to them in that medium. It was observed that the hydrogen evolved can be correlated with the nitrogen content of the proteins and the sodium employed, thus permitting the construction of a curve that is characteristic for each protein. Furthermore, it was shown that the physiological activity of a protein-like substance, such as parathyroid hormone, can be correlated with the curves mentioned (3).

The purpose of the present investigation was to make such correlations for insulin, and it was found that insulin, when treated with varying amounts of sodium in liquid ammonia, gives a protein-like curve (Fig. 1) and can therefore be classified with and compared to other proteins. It was observed, however, that insulin, unlike parathyroid hormone (3), does not give a curve representing its physiological activity, since the smallest amount of sodium added to liquid ammonia containing insulin completely inactivates the latter in respect to its power of lowering blood glucose. This is not surprising, since numerous investigators have shown that in aqueous solution insulin is easily inactivated by a variety of reducing agents (4). However, insulin may be recovered from solution in liquid ammonia without appreciable loss of its potency.

Methods

The liquid ammonia used in this work was dried by contact with metallic sodium according to the method of Fernelius and Johnson (5), and the insulin was dried in a vacuum desiccator over sulfuric acid for 6 weeks. Extensive drying controls have been made and were discussed in previous papers (2, 3).

A 0.5 gm. sample of insulin was used for each of the points on the hydrogen curve (Fig. 1), in which the atomic proportion of hydrogen evolved is plotted against the atomic proportion of sodium employed per atom of insulin nitrogen. The procedure and apparatus used to obtain these data are described in previous publications (1-3).

DISCUSSION

Reactions of Insulin with Sodium in Liquid Ammonia—The curve shown in Fig. 1 can be considered in three segments. In Segment I a rapid reduction of insulin is indicated, since only 0.1 gm. atom of hydrogen is evolved for 0.4 gm. atom of sodium added. This rapid reduction of insulin continues until about 0.8 gm. atom of sodium is added and 0.13 gm. atom of hydrogen is evolved, when the curve begins to rise, forming Segment II. During this time there is very little reduction and absorption, and the insulin is acting as an acidic substance. Here the hydrogen evolved to the sodium added has about a 1:1 ratio. Later the curve flattens out, indicating that both reduction and salt formation with the evolution of hydrogen have ceased, as is shown in Segment III.

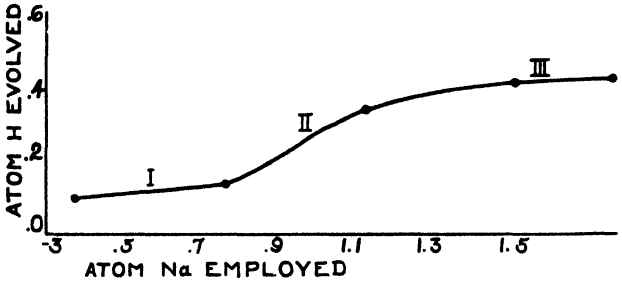


FIG. 1. Acidic hydrogen from insulin. Segment I, region of rapid reduction and absorption; Segment II, region of rapid evolution of acidic hydrogen; Segment III, region of completed reduction and slow evolution of hydrogen.

In general the curve obtained by our method for insulin resembles the curves obtained by us for other proteins in contradistinction to the curves obtained for peptones, diketopiperazine, or dipeptides such as glycylglycine. It is also apparent from these data that insulin has no prosthetic group that has a catalytic effect for the action of sodium on liquid ammonia like that shown by hematin in hemoglobin. We have shown in other work (6) that the curves for hematin and hemoglobin are linear, and continue indefinitely with the addition of sodium. This indicates a catalytic action.

The amount of reduction, as shown in Segment I, is much greater than that found for such proteins as edestin and casein, but resembles somewhat the amount of reduction found for egg albumin. However, the time required for the fading of the blue color caused by the addition of 0.1 gm. pieces of sodium was less for insulin than for other proteins which we have studied. This indicates that insulin is more easily reduced, as might be expected from its high cystine content and accompanying disulfide linkages. It has been shown by others (7) and by ourselves (1) that disulfide linkages can be reduced by metallic sodium in liquid ammonia. The total hydrogen evolved by insulin, however, is almost identical with that of casein, being more than that of egg albumin, but less than that of edestin or silk fibroin.

The solubility of insulin in liquid ammonia has been reported previously by du Vigneaud, Fitch, Pekarek, and Lockwood (8) and also by Kharasch (9) who advocated the use of liquid ammonia for the purification of crude insulin. None of the above authors indicates the extent to which insulin is soluble in liquid ammonia. Kharasch (9) extracted 10 gm. of commercial insulin (15 I.U. per mg.) with 150 cc. of liquid ammonia. He does not state the yield obtained from the soluble fraction, but claims that from it an isoelectric precipitate of high potency (24 to 27 I.U. per mg.) was obtained. In our studies we were interested in comparing the solubilities of amorphous and crystalline insulin in liquid ammonia with the solubilities of other proteins we have studied.

Most proteins and peptones[1] flocculate at once on being added to liquid ammonia. In this connection it is interesting to note that hemoglobin is essentially insoluble in liquid ammonia, but after 20 to 30 minutes the liquid ammonia becomes reddish purple in color, due possibly to the splitting off of hematin which is very soluble in this medium. On the other hand amorphous insulin (20 units per mg.) does not flocculate immediately when 0.5 gm. of it is dissolved in 200 cc. of liquid ammonia, but forms what appears to be an opalescent solution, resembling egg albumin in water. In about 45 minutes, however, flocculation sets in and a light precipitate settles out. Since this was unusual, a solubility determination was made upon crystalline insulin, with smaller quantities but the same proportions as for amorphous insulin; that is, 25 mg. of crystalline insulin and 10 cc. of liquid ammonia. In these proportions crystalline insulin is immediately soluble, forming a clear, transparent solution, and flocculation does not occur or is only faintly appreciable after several hours. In this respect crystalline insulin resembles many amino acids. With the exception of glutamic acid all of the amino acids[2] used in this work are very soluble in liquid ammonia. Both glutamic acid and glutathione form heavy viscous oils in liquid ammonia. The dipeptides, glycylglycine, glycyl-*dl*-alanine, and *dl*-leucylglycine, are very soluble in liquid ammonia, as are also the amino acids, histidine and tryptophane. However, arginine and glycine anhydride are insoluble in liquid ammonia.

---

[1] Specific examples are casein, edestin, egg albumin, parathyroid hormone, silk fibroin, sheep wool, gelatin, Witte's peptone, Armour's meat peptone, Merck's meat peptone, and Hoffmann-La Roche silk peptone.

Only two vegetable proteins have been reported as being soluble in liquid ammonia; namely, zein and gliadin (10). This observation was checked by ourselves, and we found that zein and gliadin are soluble in liquid ammonia after having been washed repeatedly with absolute ethyl alcohol and dried in a vacuum desiccator over sulfuric acid. After further drying in an oven at 80°, however, these proteins were found to be practically insoluble in liquid ammonia (2). Not knowing just how the crystalline insulin had been dried before reaching us, we subjected it to further drying in an oven at 80° for 24 hours. This treatment did not decrease its solubility in liquid ammonia.

We have shown (11) that hematin and glycine, both very soluble in liquid ammonia, react in it to form an insoluble compound which, however, is soluble in aqueous solution at pH 7.42, and has absorption bands similar to hematin. Crystalline insulin resembles hematin in that it is soluble in liquid ammonia and reacts with glycine to form a precipitate in liquid ammonia. When crystalline insulin (25 mg.) is mixed with glycine (25 mg.) and added to 10 cc. of liquid ammonia the mixture goes into solution at once. However, within a few minutes a flocculent precipitate begins to form and slowly settles out. The nature of this product is under investigation.

---

[2] It has been previously reported by du Vigneaud, Audrieth, and Loring (7) that cystine is soluble in liquid ammonia.

SUMMARY

1. Curves plotted for the acidic hydrogen evolved from insulin when it is treated with sodium in liquid ammonia resemble curves obtained for casein, egg albumin, edestin, and silk fibroin.

2. Insulin has no prosthetic group that has a catalytic effect for the action of sodium on liquid ammonia such as that shown by hematin in hemoglobin.

3. The slightest reduction of insulin by sodium in liquid ammonia entirely obliterates its power of lowering blood glucose.

4. Crystalline insulin is soluble in liquid ammonia after having been dried in an oven at 80°.

5. Insulin, dissolved or dispersed in liquid ammonia, can remain in contact with it for 24 hours without apparent inactivation.

6. Crystalline insulin forms an insoluble precipitate with glycine in liquid ammonia.

The author wishes to thank Eli Lilly and Company for the amorphous insulin and Dr. H. Jensen of the Johns Hopkins University for the crystalline insulin used in this work.

BIBLIOGRAPHY

1. Miller, C. O., and Roberts, R. G., *J. Am. Chem. Soc.*, **56**, 935 (1934).
2. Roberts, R. G., and Miller, C. O., *J. Am. Chem. Soc.*, **58**, 309 (1936).
3. Roberts, R. G., Tweedy, W. R., and Smullen, G. H., *J. Biol. Chem.*, **112**, 209 (1935-36).
4. Jensen, H. F., Insulin, New York, 62-65 (1938).
5. Fernelius, W. C., and Johnson, W. C., *J. Chem. Education*, **6**, 444 (1929).
6. Roberts, R. G., and Miller, C. O., *Proc. Soc. Exp. Biol. and Med.*, **31**, 522 (1934).

7. du Vigneaud, V., Audrieth, L. F., and Loring, H. S., *J. Am. Chem. Soc.*, **52,** 4500 (1930).
8. du Vigneaud, V., Fitch, A., Pekarek, E., and Lockwood, W. W., *J. Biol. Chem.*, **94,** 233 (1931-32).
9. Kharasch, M. S., U. S. patent 1,866,569; *Chem. Abst.*, **26,** 4682 (1932).
10. Taft, R., *Tr. Kansas Acad. Sc.*, **32,** 38 (1929); *J. Physic. Chem.*, **34,** 2722 (1930).
11. Roberts, R. G., and Horvitz, H. J., *Tr. Illinois Acad. Sc.*, **31,** No. 2 (1938).

STUDIES IN PROTEIN METABOLISM

IX. THE UTILIZATION OF AMMONIA BY NORMAL RATS ON A STOCK DIET*

By D. RITTENBERG, RUDOLF SCHOENHEIMER, AND ALBERT S. KESTON

(*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, March 22, 1939)

It has been shown in a recent publication (1) that immature rats kept on a protein-low diet with an addition of large quantities of ammonium citrate may utilize some of the added ammonia for amino acid formation. The reaction was followed by labeling the dietary ammonia with N^{15} and determining the isotope concentration of a number of pure amino acids isolated from the body proteins. In that experiment the conditions were not physiological. The greater part of the dietary nitrogen was given as ammonium citrate and the animals lost weight during the experimental period. It was therefore difficult to draw conclusions as to the fate of ammonia in the normal metabolism.

In the present study the fate of a small amount of ammonia was followed in adult rats kept otherwise under normal conditions. They were given an ordinary stock diet, the protein of which consisted of casein. Isotopic ammonium citrate was added to the diet, the nitrogen of which corresponded to only 5.8 per cent of the total nitrogen of the diet. The rats were on this diet for 9 days and their weight remained constant. They were killed by exsanguination, and the proteins of the liver, of the walls of the intestinal tract, of the kidneys, and of the muscles were investigated separately. All these proteins contained significant amounts of isotope. The proteins were hydrolyzed; arginine, glutamic acid, aspartic acid, and glycine were isolated from the

---

* This work was carried out with the aid of grants from the Rockefeller Foundation and the Josiah Macy, Jr., Foundation.

liver protein. The same amino acids, with the exception of aspartic acid, were isolated from the wall of the intestinal tract, and arginine was isolated from the kidney. All these compounds contained some labeled nitrogen. The protein of the liver and all of the fractions isolated from this protein, contained a higher concentration of isotope than did those of the other organs. Of the amino acids isolated, glutamic acid had the highest concentration of isotope, a finding in accord with our earlier finding on feeding ammonia (1), or isotopic tyrosine (2). It gives support to the theory of von Euler and collaborators (3) according to which glutamic acid is the first amino acid to be formed from ammonia, its nitrogen being secondarily transferred to other amino acids by coupled deamination and amination according to the scheme of Braunstein and Kritzmann (4).

The small amounts of ammonia present in the food of our rats cannot have markedly altered the characteristics of our stock diet. The absorption of small amounts of ammonia (derived from amide nitrogen or putrefaction) probably occurs normally.

The experiment clearly demonstrates the utilization of ammonia for amino acid formation in normal animals, and gives support to the frequently discussed hypothesis that ammonia is an intermediate in the process.

The isotope content of the non-protein nitrogen from the different organs was only slightly higher than that of the proteins and of the "amide nitrogen" of the proteins. Hemin from the blood cells and creatine from the muscles showed an isotope content only just above the normal value. The finding with creatine is in agreement with our earlier results on feeding ammonia.

EXPERIMENTAL

Two male rats weighing 460 and 400 gm. were kept on the following diet: casein 15 per cent, corn-starch 68 per cent, yeast 5 per cent, salt mixture (5) 4 per cent, cod liver oil 2 per cent, and Wesson oil 6 per cent. To this diet was added an amount of ammonium citrate corresponding to 26.4 mg. of nitrogen per day per animal for a period of 9 days. The ammonium citrate contained 2.00 atom per cent N^{15} excess. The animals consumed an average of 22 gm. of dry diet per animal per day, corresponding to 538 mg. of total N. The total nitrogen of the diet was calculated to contain about 0.11 atom per cent N^{15} excess. A sample of the combined urine of the last day was collected. The total nitrogen contained 0.071 and the urea, isolated as the dixanthydryl derivative, contained 0.065 atom per cent N^{15} excess. The animals were killed at the end of the 9th day by exsanguination through heart puncture. Blood corpuscles, livers, kidneys, intestinal walls, and part of the total muscles were worked up separately.

Isolation of Hemin from Erythrocytes—Sodium oxalate was added to the blood during collection; the corpuscles were centrifuged off and washed once with saline solution. 5 cc. of corpuscles were hemolyzed with 10 cc. of distilled water and added dropwise with stirring to 30 cc. of hot (95°) acetic acid containing a small amount of NaCl. After cooling to 55° the hemin crystals were centrifuged off and washed with 1 per cent HCl. They were purified by dissolving in 0.3 cc. of pyridine and 0.6 cc. of chloroform. A small amount of undissolved material was centrifuged off. The clear solution was added dropwise to a boiling mixture of 10 cc. of acetic acid and 0.1 cc. of concentrated HCl. The crystals formed after 24 hours in the refrigerator were washed with 1 per cent HCl. Yield 17 mg. N found 9.0 per cent, calculated 9.1 per cent. The hemin contained 0.010 atom per cent N^{15} excess.

Treatment of Tissues

Non-Protein Nitrogen—The intestinal tracts (from the pylorus to the anus) were opened with scissors, and the contents carefully removed by washing with large volumes of water. All the organs investigated were minced first with scissors, and then ground in a porcelain mortar and extracted three times with large quantities of 6 per cent trichloroacetic acid to remove non-protein nitrogen. Samples of the non-protein nitrogen and the protein nitrogen were used for isotope analysis. Creatine was isolated from the trichloroacetic acid extract of the muscles as potassium creatinine picrate, as described before (1). The product, 2.02 gm., was assayed colorimetrically for its creatine content; found 18.2 per cent, calculated 18.6 per cent. N (Kjeldahl after reduction with Sn and HCl), found 20.5 per cent, calculated 20.7 per cent. The isotope analysis was carried out after decomposition of the picrate with H_2SO_4 and removal of picric acid by ether extraction. The creatine contained 0.009 atom per cent N^{15} excess.

Protein Nitrogen—The proteins of the organs of the two animals, livers (660 mg. of N), intestinal walls (295 mg. of N), and kidneys (130 mg. of N), were hydrolyzed with HCl and the different fractions or amino acids secured according to general methods described before (1, 2). The adaptation of these methods to the semimicroprocedures used in the present work will be described in a forthcoming publication. Glutamic acid and glycine (as hippuric acid) were isolated from the liver, as well as from the intestinal wall. Glutamic acid from liver gave N (Kjeldahl) 9.43, from intestine 9.38, calculated 9.52. Hippuric acid was recrystallized until the melting point was 189°. Arginine was isolated from the proteins of liver, intestine, and kidneys, as the flavianate. The flavianates were recrystallized from water and decomposed by butyl alcohol extraction of the acidified flavianate solution. The isotope analysis of arginine was carried out with the aqueous layer. Aspartic acid was isolated from the liver proteins. N (Kjeldahl) found 10.40, calculated 10.53. The determination of the nitrogen isotope in all fractions was carried out according to the method previously reported (6).

TABLE I

N^{15} Concentration in Nitrogen of Protein Constituents of Rats Given Ammonium Citrate (N^{15})

Fraction	Atom per cent excess in			
	Liver	Wall of intestinal tract	Kidney	Muscle
Non-protein nitrogen	0.033	0.022	0.022	0.011
Protein nitrogen	0.022		0.020	0.012
Amide nitrogen of protein	0.022	0.019	0.019	
Arginine	0.026	0.013	0.038	
Combined dicarboxylic acids as barium salts	0.035	0.011	0.015	
Glutamic acid	0.046	0.022		
Aspartic "	0.031			
Protein after removal of amide N, arginine, and dicarboxylic acids	0.014	0.009		
Glycine	0.019	0.011		

The results are given in Table I.

SUMMARY

Two adult rats were kept for 9 days on a normal stock diet containing 15 per cent casein with an addition of 26.4 mg. of ammonia nitrogen, in the form of ammonium citrate, per rat per day. The ammonia nitrogen, which represented 5.8 per cent of the total dietary nitrogen, was labeled by the isotope N^{15}.

The livers, the intestinal tracts, the kidneys, and the muscles were worked up separately. The proteins separated from the non-protein nitrogen fraction contained significant amounts of isotope. The proteins were hydrolyzed and various amino acids were isolated: arginine samples were obtained from all three proteins, glutamic acid and glycine from liver and from intestine, and aspartic acid only from liver. All these pure amino acids contained labeled nitrogen.

Creatine was isolated from muscles and hemin from erythrocytes. Both compounds contained an excess of isotope outside the limit of error of the analysis.

The experiment clearly indicates that normal rats can utilize dietary ammonia for amino acid formation.

BIBLIOGRAPHY

1. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
2. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **127**, 333 (1939).
3. von Euler, H., Adler, E., Guenther, G., and Das, N. B., *Z. physiol. Chem.*, **254**, 61 (1938).
4. Braunstein, A. E., and Kritzmann, M. G., *Enzymologia*, **2**, 129 (1937).
5. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
6. Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, **127**, 291 (1939).

THE STRUCTURE OF THE ALDOBIONIC ACID FROM FLAXSEED MUCILAGE

BY R. STUART TIPSON, CLARENCE C. CHRISTMAN, AND P. A. LEVENE

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, March 22, 1939)

Indications of the presence of an aldobionic acid in the hydrolysate from flaxseed (linseed) mucilage were first obtained by Hilger[1] in 1903, and later confirmed by Neville.[2]

Anderson and Crowder[3] succeeded in isolating the aldobionic acid, as its calcium and barium salts, and found it to be a compound of galacturonic acid with *l*-rhamnose. The *l*-rhamnose was definitely identified but the other moiety was only recognized as galacturonic acid. It remained for Niemann and Link[4] to prove conclusively that it was actually the *d* form of the uronic acid.

By oxidation with hypoiodite, Anderson and Crowder transformed the aldobionic acid to the corresponding dibasic acid, which still contained uronic acid (as revealed by the naphthoresorcinol test), and they were therefore able to assign to the aldobionic acid the structure of a galacturonido-*l*-rhamnose, tentatively suggesting that the biose link is at position 4 of the rhamnose residue, since position 6 is excluded and other positions of combination were unknown at that time.

Examples of linkage at positions 2, 3, and 5 are now known[5-7] to occur in natural substances built up of sugar or uronic acid residues, or of both types of residue. Further investigation of the structure of the aldobionic acid was therefore desirable.

---

[1] Hilger, A., *Ber. chem. Ges.*, **36**, 3197 (1903).

[2] Neville, A., *J. Agric. Sc.*, **5**, 113 (1913); *Chem. Trade J.*, 265 (1911).

[3] Anderson, E., and Crowder, J. A., *J. Am. Chem. Soc.*, **52**, 3711 (1930).

[4] Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 205 (1934).

[5] Hirst, E. L., and Jones, J. K. N., *J. Chem. Soc.*, 1174 (1938).

[6] Percival, E. G. V., and Somerville, J. C., *J. Chem. Soc.*, 1615 (1937).

[7] Haworth, W. N., Raistrick, H., and Stacey, M., *Biochem. J.*, **31**, 640 (1937).

The aldobionic acid was prepared from flaxseed mucilage by an improvement of the method of Anderson and Crowder,[3] and the product then completely methylated to the methyl ester of the pentamethyl methylaldobionide. This fully methylated derivative consisted of a syrupy mixture of α and β forms of the glycoside (*i.e.*, of the rhamnose moiety), but it was possible to isolate one form as crystalline material having a melting point of 93–94°.

On hydrolysis with mineral acid, the syrupy and the crystalline portions gave rise to equimolecular proportions of the same two products; namely, a crystalline trimethyl galacturonic acid and a crystalline dimethyl rhamnose.

The methylated uronic acid was recognized as *2,3,4-trimethyl d-galacturonic acid*[8] since, on condensation with methanol containing dry hydrogen chloride, it yielded the crystalline methyl ester of 2,3,4-trimethyl α-methyl-*d*-galacturonide previously described.[9]

It seemed plausible that the dimethyl rhamnose moiety might be either 2,3- or 3,4-dimethyl rhamnose. Negative evidence, suggesting the latter as the structure, was derived from the observation that the sugar showed no change in specific rotation on preservation of a solution of it in methanol containing 1 per cent of dry hydrogen chloride, during 20 hours at room temperature. The identity of the dimethyl rhamnose was established by comparison of its properties with those of an authentic specimen of the *3,4-dimethyl l-rhamnose* previously known.[10] They had the same specific rotation and melting point, and a mixture of the two showed no depression of melting point. The identification was confirmed by transformation of the dimethyl rhamnose (from flaxseed mucilage) to the corresponding lactone. This was the same as the 3,4-dimethyl δ-rhamnonolactone previously known.[10]

---

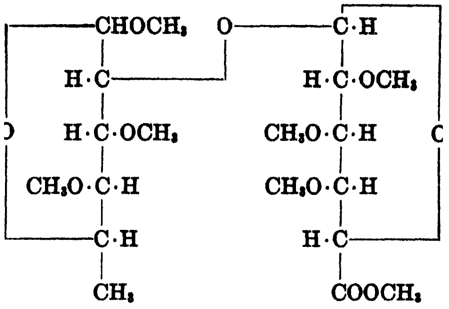
[8] Tipson, R. S., *J. Biol. Chem.*, **125**, 341 (1938).

[9] Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **120**, 597 (1937). Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937–38).

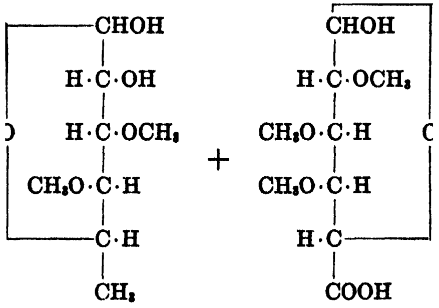
[10] Haworth, W. N., Hirst, E. L., and Miller, E. J., *J. Chem. Soc.*, 2469 (1929).

Further oxidation, with nitric acid, led to a dimethoxy hydroxyglutaric acid (isolated as its crystalline dimethylamide), analogous to that described by Baker and Haworth[11] as formed by nitric acid oxidation of trimethyl *l*-arabofuranose.

These observations clearly show that the uronic acid residue is the pyranose form of *d*-galacturonic acid and that, in the aldobionic acid, this is attached by a glycosidic link to position 2 of the rhamnose residue. The aldobionic acid from flaxseed mucilage may therefore be formulated as 2-(*d*-galacturonopyranosido)-*l*-rhamnose. Depending on the conditions, the rhamnose moiety is free to function either as a furanose or a pyranose.



Methyl ester of pentamethyl 2-*d*-galacturonido-methyl-*l*-rhamnoside



3,4-Dimethyl *l*-rhamnose 2,3,4-Trimethyl *d*-galacturonic aci

---

[11] Baker, S., and Haworth, W. N., *J. Chem. Soc.*, **127**, 365 (1925).

EXPERIMENTAL

Preparation of Ash-Free Flaxseed Mucilage—The whole flaxseed was soaked in distilled water for 2 days and the mucilage then separated from the seed by pressing through a wire sieve (No. 16 mesh). The mucilage was now filtered through fine cheese-cloth in order to remove particles of whole seed, precipitated with alcohol, squeezed free of solution, and soaked in alcohol until no longer sticky. The product contained a large amount of ash which was removed by the following treatment.

The dried, stringy, tough substance was dissolved in boiling 1 per cent hydrochloric acid, the solution allowed to stand during 15 minutes at room temperature, and the material then precipitated by the addition of 3 volumes of absolute ethyl alcohol. The product was a fine amorphous powder (no longer stringy), which was filtered off and dried with absolute alcohol and dry ether. The average yield from 50 pounds of whole flaxseed was about 750 gm.

Hydrolysis of Ash-Free Flaxseed Mucilage with Dilute Sulfuric Acid—A mixture of 1.5 kilos of dry, powdered, ash-free flaxseed mucilage with 1 liter of charcoal was added to 12 liters of 4 per cent sulfuric acid and the suspension was heated on a steam bath during 9 hours. The hot mixture was then treated with hot barium hydroxide solution until no longer acid to Congo red but acid to blue litmus. The remainder of the acid was neutralized with barium carbonate. The mixture was now filtered and the precipitate stirred for 1 hour with 8 liters of hot water and the suspension filtered.

The combined filtrates were concentrated to 1.5 liters and then precipitated, with vigorous stirring, by slowly adding 3 liters of absolute ethyl alcohol (all mother liquors being saved). The precipitated syrup was dissolved in 1 liter of water and partially precipitated with 500 cc. of alcohol. The black, tarry, precipitated syrup was discarded and the material in the mother liquor precipitated with 2 liters of alcohol. The syrup was reprecipitated by dissolving in 1 liter of water and adding 2 liters of alcohol.

This purified syrup was dissolved in 500 cc. of water, stirred overnight with charcoal, filtered, and the filtrate poured *into* 2.5 liters of alcohol. The precipitated amorphous powder was centrifuged off, shaken well with absolute alcohol, and again centrifuged. The product was stirred for 1 hour with 1 liter of absolute alcohol, filtered, washed successively with absolute alcohol and dry ether, and then dried for 24 hours in the vacuum oven at 80°. Yield 105 gm. A Willstätter estimation gave an average molecular weight of 470 to 480. ($C_{12}H_{19}O_{11}Ba_{\frac{1}{2}}$, calculated 408.)

The aqueous alcohol mother liquors from the above material were concentrated to dryness, dissolved in 500 cc. of water, and precipitated with 1.5 liters of alcohol. This syrup was precipitated twice more in the same manner and then dissolved in 300 cc. of water and poured *into* 1500 cc. of alcohol. The product was now isolated as above, giving a second crop of 55 gm. of the barium salt having an average molecular weight of 450 to 460 on the basis of a Willstätter estimation.

These two fractions were then combined and used in the subsequent experiments.

When the ash-free flaxseed mucilage was hydrolyzed during 18 to 20 hours with 4 per cent sulfuric acid according to the directions of Anderson and Crowder,[8] a barium salt was obtained which had a molecular weight (by Willstätter's method) of 410, which is close to the calculated value (408). However, when this material was methylated as now described, it was found to contain 60 to 70 per cent of monose, whereas the material obtained after hydrolysis during 9 hours contained only 10 to 15 per cent of monose. The Willstätter method evidently does not give the correct value in the case of this substance and treatment during 18 to 20 hours hydrolyzes the aldobionic acid to a great extent.

Methyl Ester of Pentamethyl Methylaldobionide from Flaxseed Mucilage—15 gm. of the barium salt of the aldobionic acid from flaxseed mucilage were dissolved in 50 cc. of water and almost quantitatively freed from barium with sulfuric acid. Care was taken to leave about 2 to 3 per cent of the barium, in order to avoid the presence of free sulfuric acid. The barium sulfate was removed and the solution concentrated to dryness. The free aldobionic acid was separated from the small amount of barium salt by extracting with warm methanol, filtering, and evaporating the filtrate to dryness.

The free acid was methylated with sodium hydroxide and dimethyl sulfate according to the directions of Levene and coworkers.[12] Yield 6.1 gm. The substance was then esterified with diazomethane, methylated five times with Purdie's reagents, and the product distilled at a pressure of 0.1 to 0.2 mm. A small portion (0.5 gm.) of low boiling methylated monose derivative was obtained at 130–135°, while the main product (4.1 gm.) distilled at 165–169° (bath temperature, 170–175°). This main product was then redistilled and had a composition agreeing approximately with that calculated for the methyl ester of a pentamethyl hexuronido-methylmonodesoxyhexoside.

2.805 mg. substance: 27.08 cc. 0.01 N sodium thiosulfate
$C_{19}H_{34}O_{11}$. Calculated, OCH_3 49.55; found, OCH_3 49.91

This glassy syrup was dissolved in 30 to 40 cc. of hexane and, upon slow spontaneous evaporation, the substance crystallized. Total yield of crystals, 1.4 gm. The crystalline product had a composition agreeing with that calculated for the methyl ester of a pentamethyl hexuronido-methylmonodesoxyhexoside.

5.670 mg. substance: 10.790 mg. CO_2 and 3.990 mg. H_2O
3.227 " " : 30.92 cc. 0.01 N sodium thiosulfate
$C_{19}H_{34}O_{11}$. Calculated. C 52.02, H 7.8, OCH_3 49.55
Found. " 51.90, " 7.8, " 49.54

The substance was recrystallized many times from hexane but a constant melting point was never obtained, although the composition remained unchanged.

The crystalline methyl ester of pentamethyl methylaldobionide had a rather indefinite melting point at about 93–94° and the following specific rotation.

$$[\alpha]_D^{24} = \frac{+2.93° \times 100}{2 \times 1.129} = +129.8° \quad \text{(in water)}$$

The syrupy methyl ester had $n_D^{25} = 1.4675$.

Hydrolysis of Methyl Ester of Pentamethyl Methylaldobionide—The course of hydrolysis at 100° of a 2 per cent solution of the crystalline methyl ester of pentamethyl methylaldobionide in 7 per cent aqueous hydrochloric acid was studied polarimetrically. Under these conditions, the initial specific rotation of the solution

---

[12] Levene, P. A., Meyer, G. M., and Kuna, M., *J. Biol. Chem.*, **125**, 703 (1938). Levene, P. A., and Kuna, M., *J. Biol. Chem.*, **127**, 49 (1939).

($[\alpha]_D^{25}$ = +130.0°) showed a smooth change as follows: +119.6° (30 minutes), +111.2° (1 hour), +97.0° (2 hours), +86.4° (3 hours), +78.5° (4 hours), +73.4° (5 hours), +71.3° (6 hours), constant thereafter. (The rotation of an equimolecular mixture of 2,3,4-trimethyl galacturonic acid[8] and 3,4-dimethyl rhamnose[10] is calculated to be +70°.)

The solution, from 4 gm. of methylglycoside methyl ester, was cooled to room temperature and the hydrochloric acid neutralized by addition of silver carbonate. The precipitate was centrifuged off and washed several times by shaking with water and centrifuging.

The solution and washings were combined and hydrogen sulfide was passed into the solution until all the silver had been precipitated. The mixture was then aerated, filtered, and the filtrate evaporated under diminished pressure to a volume of 125 cc.

A slight excess of barium carbonate was now added and the mixture was warmed at 60° until the solution was neutral to litmus. The mixture was then filtered and the filtrate evaporated under diminished pressure to a yellow glass (weight, 4.5 gm.). This was thoroughly extracted with fifteen portions (100 cc. each) of boiling dry ether under a reflux condenser. The ether extracts were united and evaporated to dryness under diminished pressure, giving a colorless crystalline mass (weight, 1.5 gm.). This crystalline material was recrystallized from ether-pentane and then had a melting point of 94–95°, n_D^{25} = 1.4711 (in the superfused state), and the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.36° \times 100}{2 \times 0.990} = +18.2° \quad \text{(equilibrium, in water)}$$

It had the following composition.

5.402 mg. substance: 9.890 mg. CO_2 and 4.070 mg. H_2O
3.889 " " : 24.22 cc. 0.01 N sodium thiosulfate

$C_8H_{16}O_5$.	Calculated.	C 49.97,	H 8.4,	OCH_3 32.30
	Found.	" 49.92,	" 8.4,	" 32.20

The properties previously recorded[10] for authentic 3,4-dimethyl rhamnose are, m.p. = 91–92° and $[\alpha]_D^{20}$ = +18.6° (equilibrium, in water).

In methyl alcohol containing 1 per cent of dry hydrogen chloride the sugar had $[\alpha]_{\mathrm{D}}^{22} = -8.8°$ ($c = 1.02$), and this value remained constant during several hours at room temperature, indicating that there was no free hydroxyl group at carbon atom (4).

After removal of the dimethyl rhamnose, by extraction with ether, the residue consisted of the barium salt of trimethyl galacturonic acid. This material was dissolved in water and the barium removed quantitatively by adding N sulfuric acid dropwise.

The barium sulfate was removed by centrifuging, the precipitate washed several times by centrifuging with water, and the combined solutions evaporated under diminished pressure to a volume of 25 cc. This solution was then extracted with four portions (50 cc. each) of chloroform, the chloroform extract dried with anhydrous sodium sulfate, filtered, and the filtrate evaporated to a colorless glass, which was obtained crystalline from ether.

As the product was difficult to purify, it was identified by transformation to its methylglycoside methyl ester.

Preparation of Methyl Ester Methylglycoside of Trimethyl Galacturonic Acid—A 1 per cent solution of the trimethyl galacturonic acid in methyl alcohol containing 1 per cent of dry hydrogen chloride was gently boiled under a reflux during 6 hours.

The solution was then cooled and rendered neutral by the addition of dry silver carbonate. The mixture was filtered and the filtrate evaporated to dryness under diminished pressure, giving a quantitative yield of colorless, glassy substance. It was readily obtained crystalline by dissolving in dry ether, filtering off a trace of insoluble material, and evaporating the filtrate in a dish at room temperature. It separated in large square platelets which were filtered off, washed with a little ether, and dried. M.p. = 70°.

It had the following composition.

5.201 mg. substance: 9.510 mg. CO_2 and 3.570 mg. H_2O
2.800 " " : 31.73 cc. 0.01 N sodium thiosulfate
$C_{11}H_{20}O_7$. Calculated. C 49.97, H 7.6, OCH_3 58.72
Found. " 49.87, " 7.7, " 58.59

Its specific rotation was as follows:

$$[\alpha]_{\mathrm{D}}^{24} = \frac{+3.58° \times 100}{2 \times 1.202} = +149.0° \quad \text{(in acetone)}$$

The properties previously recorded[9] for the methyl ester of 2,3,4-trimethyl α-methyl-*d*-galacturonide are m.p. = 70.2–70.3° and $[\alpha]_D^{27} = +149.3°$ (in acetone).

Oxidation of Dimethyl Rhamnose (from Aldobionic Acid) with Bromine—To a solution of 0.5 gm. of crystalline dimethyl rhamnose in 10 cc. of water was added 0.5 cc. of bromine. The mixture was kept in a glass-stoppered Erlenmeyer flask during 24 hours at room temperature. The brown solution was then aerated until colorless and sufficient silver carbonate was added to neutralize all the mineral acid. The mixture was filtered, the precipitate washed with water, and excess hydrogen sulfide passed into the combined filtrate and washings. The mixture was aerated, filtered, and the filtrate evaporated to dryness. The product was dissolved in dry ether, filtered from a trace of insoluble material, and the filtrate evaporated to dryness, giving a colorless, crystalline mass.

It was recrystallized by dissolving in 5 cc. of dry ether, adding 20 cc. of pentane, and allowing to stand in the refrigerator, whereupon it separated as a mat of long, fine colorless needles having a melting point of 76–77° and the following specific rotation.

$$[\alpha]_D^{24} = \frac{-0.71° \times 100}{2 \times 0.224} = -158.5° \quad \text{(initial, in water)}$$

changing to −120.5° (50 hours), and −116.1° (92 hours), constant thereafter.

It had the following composition.

5.514 mg. substance: 10.195 mg. CO_2 and 3.600 mg. H_2O
3.299 " " : 20.80 cc. 0.01 N sodium thiosulfate
$C_8H_{14}O_5$. Calculated. C 50.50, H 7.4, OCH_3 32.64
Found. " 50.43, " 7.3, " 32.59

The properties previously recorded[10] for 3,4-dimethyl δ-rhamnonolactone are m.p. = 66–68° and $[\alpha]_D^{26} = -153°$ (initial value, in water), changing to the constant value of −119° after 86.5 hours.

Oxidation of Dimethyl Rhamnose (from Aldobionic Acid) with Nitric Acid—0.45 gm. of crystalline dimethyl rhamnose (from flaxseed mucilage) was dissolved in 8 cc. of concentrated nitric acid (*d* = 1.42) and the oxidation conducted exactly as previously described for the oxidation of trimethyl xylulose.[13] The product was esterified and the ester treated with a solution of methylamine in methanol. Colorless crystals of the dimethylamide were obtained, having the following composition.

2.762 mg. substance: 4.705 mg. CO_2 and 1.900 mg. H_2O
3.300 " " : 0.332 cc. N_2 (768 mm. at 27°)
$C_9H_{18}O_5N_2$. Calculated. C 46.12, H 7.8, N 11.97
Found. " 46.45, " 7.7, " 11.57

Preparation of 3,4-Dimethyl l-Rhamnose from l-Rhamnose—Since the yield at each stage of the preparation was greatly improved by appropriate modification of the methods of earlier workers, our method will be briefly described.

50 gm. of rhamnose hydrate were suspended in 200 cc. of dry pyridine. 200 cc. of acetic anhydride were added and after about 5 minutes at room temperature, the suspension was cooled somewhat in ice. After 15 minutes the sugar had dissolved completely. The solution was then cooled to 25° and allowed to stand overnight at room temperature.

The solution was now evaporated under diminished pressure to a syrup weighing 120 gm. This was poured, with stirring, into 1 liter of filtered ice water. The precipitated syrup was dissolved in chloroform and the aqueous layer extracted several times with chloroform. The combined chloroform solution was washed successively with ice-cold dilute sulfuric acid, ice water, dilute sodium bicarbonate solution, and ice water. It was dried with anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness, giving a practically quantitative yield of syrupy tetraacetyl rhamnose. (Previously recorded yields, 75 per cent,[14] 80 to 85 per cent.[10])

The tetraacetyl *l*-rhamnose was then converted to the bromotriacetyl derivative as described for the preparation of bromotetraacetyl mannose,[15] giving a yield of 96.5 per cent of the theoretical of dry, crystalline product. (Previously recorded yields, 65 per cent,[14] 85 per cent.[10])

---

[13] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **120**, 607 (1937).

[14] Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, **53**, 2362 (1920).

[15] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **90**, 89 (1931).

By the following method, the bromoacetate was transformed to 3,4-diacetyl 1,2-methyl-orthoacetyl rhamnose, a yield of 76.5 per cent of the theoretical of dry, crystalline product being readily obtained. (Previously recorded yields, 20 to 40 per cent,[14] variable up to 50 per cent but usually about 20 per cent,[10] 45 per cent.[16])

To 10 gm. of dry, crystalline bromotriacetyl *l*-rhamnose was rapidly added a solution of 4 cc. of quinoline (dried over barium oxide) in 20 cc. of dry methanol, the mixture was rapidly swirled until all the crystals of bromoacetate had dissolved, and then the solution was kept at room temperature, with the exclusion of atmospheric moisture, during 90 minutes.

The solution was now diluted to 75 cc. with dry methanol and a considerable excess of silver acetate was added. The mixture was filtered with suction through a thin layer of silver acetate and the precipitate well washed with methanol. The combined filtrate and washings were evaporated to dryness under diminished pressure to a thick syrup, which was dissolved in chloroform, diluted to 1 liter with chloroform, and the precipitated silver acetate removed by filtration. Evaporation of the chloroform solution under diminished pressure gave a pale yellow syrup, which was dissolved in 10 cc. of warm methanol. 10 cc. of water were added to the solution and, on standing in the refrigerator, a first crop of 4 gm. of crystalline product was deposited.

The mother liquor was evaporated to dryness, dissolved in chloroform, and the chloroform solution shaken successively with water, dilute sodium bicarbonate solution, and water. It was dried with anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness. Ten separate portions (50 cc. each) of water were now added, the mixture being evaporated to dryness before each new addition. In this way, the major part of the free quinoline was removed.

The resulting syrupy product was now dissolved in a little dry ether and a large volume of pentane was added. On standing in the refrigerator, a second crop of 2.6 gm. of crystalline product settled out. The total yield of crystalline product amounted to 76.5 per cent of the theoretical.

---

[16] Haworth, W. N., Hirst, E. L., and Samuels, H., *J. Chem. Soc.*, 2861 (1931).

The 3,4-diacetyl 1,2-methyl-orthoacetyl rhamnose was then deacetylated in the usual manner, with a solution of ammonia in methanol, to give a quantitative yield of the orthoacetate. The product was methylated and hydrolyzed as described by Haworth and coworkers[10] to give 3,4-dimethyl rhamnose having a melting point of 94–95° and other properties as described by these authors.

STATISTICS OF SOME BIOCHEMICAL VARIABLES ON HEALTHY MEN IN THE AGE RANGE OF TWENTY TO FORTY-FIVE YEARS*

BY E. MORTON JELLINEK AND JOSEPH M. LOONEY

(From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester)

(Received for publication, February 16, 1939)

In the course of investigating the organic characteristics of schizophrenic patients the need for adequate control material arose. A survey of the data on control normals available in the biochemical and clinical literature led us to build up our own controls. The reasons for this are as follows: (1) For some variables the only data available on normal controls have been established by techniques which have become obsolete. (2) The normal controls most frequently referred to in the literature have been so called hospital normals, *i.e.* convalescent patients, prisoners in whom the effect of institutionalization may have been reflected, or college freshmen who are largely in the growing state. All these are biased samples.[1] (3) For some biochemical variables not more than six or eight normal controls have been tested in a given laboratory. Although determinations on twenty-five to thirty subjects could be obtained by combining the data gathered by different investigators, such combinations do not seem to be legitimate owing to the incomparability of either the biochemical techniques or of the test conditions. (4) Commonly tests on normal controls have been carried out in a period of a few weeks. When the variable under investigation is liable to seasonal fluctuations, this clustering of tests may reflect the bias of the testing season, (5) Within a given sample, testing conditions have frequently been found not to be constant. Thus some subjects

---

* This investigation was financed in part by a grant from the Rockefeller Foundation.

[1] Throughout this paper sample is used in the standard statistical sense.

were tested under basal and others under non-basal conditions. Furthermore, many reports contain insufficient information as to testing conditions. (6) With few exceptions norms have been established on one determination per subject, or repetitions when made have been reported as averages per individual, so that no estimate can be obtained of that variation which takes place within the individual himself. (7) Only rarely have more than four or five different biochemical variables been tested on the same normal subject.

Our first control subjects were volunteers from hospital physicians, research workers, medical students, and attendants. These men, who were in good health, had an age range from 20 to 45 years, and, but for the fact that most of them had more or less sedentary habits, the sample is not biased. The measurements on these volunteers, however, were unsatisfactory, inasmuch as the convenience of the volunteers had to receive much consideration. Neither was it feasible to repeat measurements of the same variable nor to obtain measurements of many variables on the same subjects. Single measurements of two or three variables were made on one group (of from twenty-five to thirty-five men) and then another group had to be recruited.

A grant from the Rockefeller Foundation made it possible to hire thirty normal controls. These men were also in the age range of 20 to 45 years. They were subjected to a searching physical as well as psychiatric examination. Only men free of any detectable organic disease and of frank neurosis were included among the experimental subjects. The normal controls lived for 8 weeks in the hospital and a large number of biochemical, clinical, and psychological tests were carried out with two to twelve repetitions on each subject. The admission of normal controls was spread over a full calendar year, and thus any possible seasonal fluctuations were compensated in the mean values.

During their stay in the hospital the activity and diet of all the men were approximately the same. All tests were made under a "basal" condition, that is, in a state of postabsorption and complete physical relaxation.

The statistics pertaining to forty biochemical variables are presented here in Table I. For estimates of the variation taking place within the individual himself, only the results on the hired "professional" subjects can be used. The number of such subjects used differs from one variable to another, since only those subjects can be considered on whom the results of determinations on all repetitions were valid. Since, however, there may be some interest in statistics from a larger sample, the data from the volunteer subjects and the "professionals" have been combined for part of the variables, and simple statistics pertaining to this merged group are given under the heading "Large sample" in Columns 2 to 5 of Table I. Merging of samples was performed only when statistical tests justified such a procedure. The more detailed statistics of the "professionals" are given under the heading "Small sample" in Columns 6 to 13 of Table I.

The standard deviation $\sqrt{S(x-\bar{x})^2/n-1}$ (Column 5) refers to the scatter of one reading per individual around the group mean. The standard deviation "between means of individuals" (Column 11) measures the scatter not of single readings but of the average readings of individuals around the group mean. The "within individuals" standard deviation (Column 12) is an estimate of the average variation of an individual around his own mean. The magnitude of this "within individuals" standard deviation should give one an idea of the *consistency* of the individual relative to the variable under investigation. However, different variables are measured in different units and thus it is not possible to compare the "within individual" standard deviation of one variable with that of another variable in order to determine which variable shows greater consistency. For this purpose some abstract value is required. In Column 13 we give such abstract values in the form of the intraclass correlation coefficient. This coefficient measures the closeness of values on repetition, and the coefficients are comparable for all variables. A coefficient of +1 would signify that all values were absolutely identical on repetition.[2] Other, and even more adequate criteria of consistency could be used, but they would require much explanation to be feasible.

While it is not intended to compare the findings presented here with findings of other laboratories, nor to discuss the contents of Table I, we should like to enlarge on some principles involved in

---

[2] The commonly used interclass correlation coefficient measures the consistency of relationship but not closeness of the values.

TABLE I

Means, Standard Deviations, and Ranges of Biochemical Variables in Normal Controls

Variable	Large sample				Small sample (various repetitions per subject)							
	No. of individuals	Mean	Range	S.D.	No. of individuals	No. of readings per individual	Time between repetitions	Mean	Range	S.D. between means of individuals	S.D. within individuals	Intraclass correlation coefficient
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
Whole blood												
							wks.					
Sugar (1),* *mg. per 100 cc.*	58	91.4	60 –111	6.2	27	4	2	88.0	60 –111	5.8	7.6	0.24
Total non-protein N (2), *mg. per 100 cc.*	61	33.8	27 – 47	2.75	30	2	6	32	27.0 – 39.7	2.20	2.60	0.19
Urea N (3), *mg. per 100 cc.*	61	15.0	10.6 – 29.0	1.60	30	2	6	14	10.7 – 20.1	1.30	1.60	0.13
Uric acid N (4), *mg. per 100 cc.*	61	3.2	1.92– 4.40	0.50	30	2	6	3.0	1.99– 4.37	0.40	0.37	0.39
Amino " " (5), " " *100* "	23	6.3	5.5 – 7.2	0.50								
Creatine " (6), " " *100* "	30	1.25	1.0 – 1.6	0.16								
Creatinine " (6), " " *100* "	59	0.92	0.5 – 1.5	0.04	30	2	6	1.33	1.01– 1.48	0.06	0.09	−0.17
Undetermined N,† *mg. per 100 cc.*	21	9.7	4.7 – 18.9	3.11								
Phosphorus (7), *mg. per 100 cc.*					27	2	6	3.5	2.78– 4.88	0.25	0.38	0.23
Chlorides (8), " " *100* "					26	2	6	462	422 –496	10	11	0.55
O_2, arterial (9), *vol.%*					28	2	6	19	10.5 – 22.8	1.5	2.0	0.37
Venous (9), *vol.%*	67	10.6	4.0 – 17.5	2.9	29	2	6	10.2	4.0 – 14.6	1.8	2.4	0.39
CO_2, arterial (9), *vol.%*					28	2	6	50	40.8 – 57.5	1.2	2.4	0.00
Venous (9), *vol.%*	67	58	49.7 – 63.5	2.8	29	2	6	58	54.9 – 61.7	1.3	2.0	0.22

Lactic acid, arterial (10), *mg. per 100 cc.*					21	2	6	10.2	6.3 - 22.9	1.28	1.72	0.36
Venous (10), *mg. per 100 cc.*	39	10.4	6.6 - 15.3	1.9	21	2	6	10.5	6.6 - 15.3	1.4	2.0	0.28
Glutathione,* total, arterial (11), *mg. per 100 cc.*					22	2	6	40	25.8 - 45.7	3.1	3.1	0.59
Total, venous (11), *mg. per 100 cc.*	40	41	30.7 - 50.8	4.0	22	2	6	40	30.7 - 50.0	2.7	2.0	0.75
Glutathione, reduced, arterial (11), *mg. per 100 cc.*					21	2	6	35.0	21.3 - 47.2	3.0	4.0	0.36
Reduced, venous (11), *mg. per 100 cc.*	39	37	26.3 - 47.9	3.9	21	2	6	36	26.3 - 45.0	2.6	7.4	−0.37
pH, arterial (12), 38.0°					16	2	6	7.49	7.34- 7.57	0.03	0.05	0.24
Venous (12), 38.0°					18	2	6	7.42	7.29- 7.52	0.04	0.05	0.07
Serum												
Total lipid (13), *mg. per 100 cc.*					21	2	1	507	385 -682	73	14	0.96
Phospholipids (13), *mg. per 100 cc.*					21	2	1	214	155 -330	36	7	0.96
Total cholesterol (14), *mg. per 100 cc.*	47	180	140 -275	28	21	2	1	186	140 -275	30	7	0.94
Free cholesterol (14), *mg. per 100 cc.*					21	2	1	52	35 - 85	11	2.6	0.94
Ester cholesterol,† *mg. per 100 cc.*					21	2	1	134	99 -190	19	5.7	0.91
Total protein (15), *gm. per 100 cc.*					23	2	6	8.1	6.20- 13.75	0.80	1.40	0.08
Albumin (15), *gm. per 100 cc.*					23	2	6	5.15	2.46- 9.21	0.60	1.30	−0.10

TABLE I—*Concluded*

Variable	Large sample				Small sample (various repetitions per subject)							
	No. of individuals	Mean	Range	S.D.	No. of individuals	No. of readings per individual	Time between repetitions	Mean	Range	S.D. between means of individuals	S.D. within individuals	Intraclass correlation coefficient
(1	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
Serum—*Concluded*												
							wks.					
Globulin,† *gm. per 100 cc.*					23	2	6	3.00	1.40– 7.67	0.80	1.00	0.44
Calcium (16), *mg. per 100 cc.*					29	2	6	10.5	9.6 – 11.8	0.30	0.50	0.18
Potassium (17), *mg. per 100 cc.*					25	2	6	19.4	14.3 – 22.0	0.73	1.81	−0.23
K:Ca ratio					25	2	6	1.85	1.30– 2.21	0.09	0.17	0.10
Ca:P product					26	2	6	37	24.19– 46.26	2.9	4.2	0.32
Choline esterase (as CO_2) (18), *c.mm. per cc.*					20	2	1	76	35 –136	24	3.4	0.97
Urine												
Total N (19, 20), *gm.*	54	11.5	6.1 – 23.4	2.3	28	4	2	11.9	6.12– 22.89	2.2	2.4	0.37
Urea N (19, 21), *gm.*	53	9.6	6.0 – 16.5	1.87	27	4	2	9.85	6.0 – 16.5	1.85	1.84	0.43
Uric acid N (19, 22), *gm.*	52	0.15	0.07– 0.25	0.06	27	4	2	0.15	0.07– 0.25	0.09	0.10	0.32
Ammonium N (23), *gm.*	52	0.42	0.14– 1.47	0.15	27	4	2	0.42	0.14– 1.47	0.11	0.15	0.21
Creatinine N (24), *gm.*	54	0.57	0.23– 0.85	0.10	28	4	2	0.57	0.23– 0.85	0.08	0.09	0.66
Residual N,† *gm.*	52	0.71	0.06– 1.80	0.24	27	4	2	0.73	0.06– 1.77	0.24	0.34	0.19

* The methods used are described in the references cited.

† By difference.

the evaluation of these statistics, as well as in the method of gathering such data.

Great caution must be exercised in using *ranges* for norms. Not infrequently ranges determined on small samples have been used as the limits of normality in clinical medicine. First, the range of a distribution is a rather unstable statistic, and second, whereas the estimates of the mean and of the standard deviations are affected in their reliability but not in their actual values by the size of the sample, the range depends largely on this factor. In Table II are given mean ranges for various sized samples.

Table II hardly requires discussion. It is evident that ranges obtained from small samples cannot serve to establish the limits of normality. Variation should generally be expressed in terms of the variance or of the standard deviation.

TABLE II

Mean Ranges of Samples of Various Sizes Drawn from a Normal Universe Having a Standard Deviation of 10 Units

This table has been computed from Table XII of Pearson (25).

Sample size	Mean range	Sample size	Mean range
2	11.28	40	43.22
3	16.93	50	44.98
4	20.59	100	50.02
5	23.26	200	54.92
10	30.08	300	57.56
20	37.35	500	60.73
30	40.85	1000	64.83

In evaluating the variation within the individual the time elapsing between repetitions must be considered. Usually one finds that the greater the time interval the greater is also the intraindividual variation. Below are given "within individual" standard deviations for oxygen consumption tests, 2 days, 3½ months, and 7 months apart.

52 individuals, 2 tests each	S.D. within individuals
	calories per 24 hrs.
2 days apart	120
3½ mos. "	138
7 " "	169

It is evident that the time interval between repetitions must be the same for all individuals and it is advisable to state the interval in connection with the "within individuals" standard deviation. The standard deviation "between means of individuals," however, is generally not affected by the time interval between repetitions.

Factors which influence the variation among individuals are test conditions such as basality or non-basality. In the latter, although the group mean may not be affected, the variation from subject to subject is usually increased owing to the differing degrees of non-basality. For venous lactic acid we have found in the basal state a standard deviation among individuals of 4.6 mg. per 100 cc., and in the non-basal state a standard deviation of 7.1. Reduction of the standard deviation "between individuals" is important, since it increases the reliability of the mean value.

Another important point is that in estimating the "within individuals" standard deviation it is necessary to determine whether a systematic shift has occurred between the means of repetitions. Such a shift may be due to season, but it may be also due to habituation to the test. The latter is the case with variables which are influenced by emotion. The blood sugar during fasting may show such a habituation trend on repetition. If a trend is present, the "within individuals" standard deviation may become exaggerated. If this trend is significant, one may correct for the variation due to the trend among the means of repetitions and determine the residual variation. This residual variation may be regarded as the "net within" variation.

The standard deviations in Columns 11 and 12 of Table I have been derived from estimates of the "between individuals" and "within individuals" variances respectively, isolated in analyses of variance.[3] In order to transform the "between individuals" variance into the variance between the *means* of individuals, the former variance was divided by the number of readings per individual (Column 7). The square root of this adjusted variance is the standard deviation between the *means* of individuals. The "within individuals" variance as obtained in the analysis of variance naturally does not require any adjustment, and its square root is the "within individuals" standard deviation. Thus, by retracing these steps the standard deviations of Columns 11 and 12 can be transformed back into the original estimates of the analysis of variance, and tests of homogeneity, that is, tests of the significance of the difference of "between" and "within" individuals variances, can be performed. Since the number of individuals as well as the number of readings per individual is reported in Columns 6 and 7, the "degrees of freedom" are easily computed, and the significance of the differences or of the ratios of the variances can be tested by using the appropriate tables.

---

[3] The formula for the "between individuals" variance is $n\overset{k}{\underset{1}{S}}(\bar{x}_k - \bar{x})^2/k - 1$; this divided by n is the variance between the *means* of individuals and thus the standard deviation between means of individuals is $(\overset{k}{\underset{1}{S}}(\bar{x}_k - \bar{x})^2 / k - 1)^{\frac{1}{2}}$. The "within individuals" variance is $\overset{k}{\underset{1}{S}}\overset{n}{\underset{1}{S}}(x - \bar{x}_k)^2 / k(n - 1)$. k denotes the number of classes of individuals and n denotes the number of observations per class or per individual, so that kn is the total number of observations; x is any observation; $\bar{x}$ is the mean of the kn readings and $\bar{x}_k$ is the mean of any given class or individual. $\overset{k}{\underset{1}{S}}$ stands for

BIBLIOGRAPHY

1. Folin, O., and Wu, H., *J. Biol. Chem.*, **41**, 367 (1920). Folin, O., *J. Biol. Chem.*, **82**, 83 (1929).
2. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
3. Looney, J. M., *J. Biol. Chem.*, **88**, 189 (1930).
4. Folin, O., *J. Biol. Chem.*, **86**, 179 (1930).
5. Folin, O., *J. Biol. Chem.*, **51**, 377 (1922).
6. Folin, O., *J. Biol. Chem.*, **17**, 475 (1914).
7. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
8. Whitehorn, J. C., *J. Biol. Chem.*, **45**, 449 (1921).
9. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
10. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, **73**, 335 (1927).
11. Woodward, G. E., and Fry, E. S., *J. Biol. Chem.*, **97**, 465 (1932).
12. Stadie, W. C., O'Brien, H., and Laug, E. P., *J. Biol. Chem.*, **91**, 243 (1931).
13. Bloor, W. R., *J. Biol. Chem.*, **77**, 53 (1928); **82**, 273 (1929).
14. Turner, M. E., *J. Biol. Chem.*, **92**, 495 (1931).
15. Howe, P. E., *J. Biol. Chem.*, **49**, 109 (1921).
16. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, **63**, 461 (1925).
17. Breh, F., and Gaebler, O. H., *J. Biol. Chem.*, **87**, 81 (1930).
18. Ammon, R., *Arch. ges. Physiol.*, **233**, 486 (1933).

---

summation over all classes or individuals, and $\overset{n}{\underset{1}{S}}$ stands for summation over all values within a given class or individual.

19. Folin, O., Laboratory manual of biochemistry, New York and London, 5th edition (1934).
20. Folin, O., and Denis, W., *J. Biol. Chem.*, **26**, 486 (1916).
21. Folin, O., and Denis, W., *J. Biol. Chem.*, **26**, 501 (1916). Folin, O., and Youngburg, G. E., **38**, 111 (1919).
22. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 459 (1919).
23. Folin, O., and Bell, R. D., *J. Biol. Chem.*, **29**, 329 (1917).
24. Folin, O., *J. Biol. Chem.*, **17**, 469 (1914).
25. Pearson, K., Tables for statisticians and biometricians, London, pt. 2 (1931).

RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

VI. THE PHOSPHOLIPID METABOLISM OF NEOPLASTIC TISSUES (MAMMARY CARCINOMA, LYMPHOMA, LYMPHOSARCOMA, SARCOMA 180)

By H. B. JONES, I. L. CHAIKOFF, and JOHN H. LAWRENCE

(From the Division of Physiology of the Medical School and the Radiation Laboratory, University of California, Berkeley)

(Received for publication, March 14, 1939)

Attempts to determine the rate of phospholipid *turnover* in tumors have been few, owing to the lack of adequate methods for such studies. Employing elaidic acid as a tagged fat, Haven (1) showed that the rate of incorporation as well as loss of this fatty acid occurs at a slower rate in tumor (Carcinosarcoma 256) phospholipid than in liver phospholipid. As shown in previous reports from this laboratory (2–4), radioactive phosphorus provides a new means of identifying the rate of phospholipid turnover in a tissue or organ. This method has been employed here for measuring phospholipid metabolism in tumors. Four types of mouse tumors were investigated, namely a mammary carcinoma, a lymphosarcoma, a lymphoma, and Sarcoma 180, for it seems likely, in view of the different cell types involved in such growths, that no single type can provide an index for all tumor activity.

EXPERIMENTAL

The data recorded here were obtained from separate analyses of one or more tissues removed from 313 mice. Both normal and tumor-bearing animals were studied, the latter being produced by bilateral inoculation of tumor particles in the axillary region by the trocar method. The various groups of mice examined are shown in Table I. The animals were permitted access to food and water at all times in the course of the experiment. The diet consisted of a balanced mixture in pellet form, which was supple-

TABLE I

Summary of Tumor Experiments

Tissue	Group No.	Symbol in chart	Fig. No.	No. of mice used	Weight* of mice		Phosphorus injected per mouse			Tumor			Liver weight		Mouse strain
					Range	Average	Volume	P as Na_2HPO_4		Weight		Age†	Range	Average	
										Range	Average				
					gm.	*gm.*	*ml.*	*mg.*	*microcuries*	*gm.*	*gm.*	*days*	*gm.*	*gm.*	
Normal liver	1	⊕	1	20	16–26	20	0.10	0.30	6				0.86–1.45	1.03	A
" "	2	◒	1	40	16–28	21	0.20	0.60	4				0.75–1.50	1.14	ABC
" "	3	◑	1	20	25–40	33	0.30	0.90	4				0.95–1.50	1.25	A
" muscle	3	□	1	20	25–40	33	0.30	0.90	4						"
Mammary carcinoma	4	●	2	20	16–26	20	0.10	0.30	6	0.04– 0.20	0.23	9‡	0.86–1.45	1.03	"
	5	■	2	30	17–33	24	0.30	0.90	5	0.70– 2.90	1.70	28‡	0.95–1.80	1.40	"
Lymphoma	6	⦶	3	40	16–28	21	0.20	0.60	4	0.40– 1.00	0.70	14‡			ABC
	7	○	3	30	28–40	34	0.20	0.60	2	1.00– 2.80	1.70	24‡	1.40–2.60	1.90	"
Lymphosarcoma	8	⊙	4	45	25–40	33	0.30	0.90	4	2.00–10.00	6.30	21§	0.80–1.50	1.16	A
	9	⊡	4	11	28–32	30	0.20	0.60	8	0.05– 0.10	0.07	11‡			"
Sarcoma 180	10	◉	5	37	15–25	20	0.20	0.60	8.5	0.30– 2.80	1.20	15§	1.00–2.00	1.45	"

* At the time of removal of the tissues for analysis.

† Time after implantation of tumors.

‡ All injections were made at the age recorded and the tumors were removed at various intervals thereafter.

§ The phosphorus injections were made at various stages in tumor development so that all tumors when excised were of the age recorded.

mented daily with whole oats. It has previously been shown that this diet permits normal growth and development in the strains of mice employed. At various times after tumor inoculation, each mouse received intraperitoneally 0.1 to 0.3 cc. of an isotonic solution of labeled Na_2HPO_4 containing 3 mg. of phosphorus per cc. The animals were sacrificed at intervals thereafter and the tissues removed for analysis.

The following neoplasms were used: (1) a mammary carcinoma, (2) a lymphoma, (3) a lymphosarcoma, and (4) Sarcoma 180. The first three tumors were transplanted into mice of the genetically uniform A and ABC strains. It has been shown here that takes always result in these strains, that regressions do not occur, and that death of the animal invariably ensues several weeks after inoculation. Sarcoma 180, which was transplanted into a Swiss strain of mice, occasionally showed regressions. The lymphosarcoma remains localized and never shows metastases. Sarcoma 180 rarely metastasizes, while the mammary carcinoma produces visceral metastases in about 18 per cent of the cases. Lymphoma remains localized in the early periods after inoculation but later gives rise to generalized lymphomatosis and leucemia.

Treatment of Tissues—The bilateral tumors were carefully dissected and freed from all surrounding tissues. The two tumor masses removed from each mouse were placed on fat-free blotting paper and their interiors exposed. After the rapid removal of all necrotic[1] or extraneous tissues, the tumors were firmly pressed between blotting paper to remove excessive fluids. Immediately thereafter they were placed in 95 per cent ethyl alcohol and the phospholipids extracted in a manner previously described (5). The determination of the radioactivity of this lipid fraction has also been noted elsewhere (2).

---

[1] A variable tendency towards necrosis was observed in the four tumors investigated. In addition to degenerative changes, the mammary carcinoma was characterized by the formation of cysts containing white caseous material. The lymphoma showed little if any tendency towards necrotic change in its early stages, although extensive metastases were found in the axillary, pelvic, and inguinal glands. Lymphosarcoma also failed to show necrotic change in the early stages; in the older tumors, however, soft necrotic areas occurred in the center. Sarcoma 180 showed regressive as well as degenerative changes.

Phospholipid Turnover of Liver and Muscle in Normal Mice

To obtain the relative phospholipid activity of the four types of tumors examined, it seemed desirable to compare them with normal tissues as well as with each other. Two normal tissues were chosen for this purpose, namely liver and muscle, since it was previously demonstrated with radioactive phosphorus that these tissues represent high and low phospholipid activity, respectively (2, 6). Fig. 1 shows the phospholipid activity of these two tissues in *normal* mice. The values were obtained from 100 normal animals. As previously noted in the rat (2), the liver is characterized by a rapid rise in its content of labeled phospholipid, reaching a maximum very early after the administration of the radioactive phosphorus. Muscle showed a much lower activity but continued to gain labeled phospholipid through the entire period of observation, 150 hours. The similarity in the shape and height of the curves obtained for livers of both mice and rats is indeed striking. In the rat, the maximum amount of labeled phospholipid, namely 3 per cent, was deposited in the liver within 10 hours after ingestion of the phosphorus. In the mouse, the height of the curve was observed 10 hours after the intraperitoneal injection of the labeled phosphorus. The maximum amount of labeled phospholipid deposited in the animal was close to 3 per cent of the administered phosphorus per gm. of liver.

Mammary Carcinoma

The transmissible adenocarcinoma used here was first induced in mice of the A strain of Strong (7) by Gardner *et al.* (8) after prolonged folliculin administration. This neoplasm is a rapidly growing one and is associated with late visceral metastases in about 18 per cent of the cases (9). It grows in cords which tend toward gland formation and is indistinguishable histologically from the adenocarcinoma of the breast that occurs spontaneously in this strain of mice.

The phospholipid activity of this tumor is shown in Fig. 2. Two groups of mice (Table I) were studied; in one the tumors were 9 days old when the labeled phosphorus was injected, whereas the data shown in the lower curve were obtained from tumors 28 days old at the zero time of Fig. 2. Mammary carcinoma is characterized by a fairly high phospholipid activity. The maximum amount of labeled phospholipid deposited by the younger tumor was a little more than one-third the maximum amount observed for the livers of *normal* mice, but many times that found in their skeletal muscle (Fig. 1). The early rise and fall—the maximum amount of labeled phospholipid was found about 10 hours after the P^{32} administration—definitely show that the phospholipid activity of this type of tumor bears a greater resemblance to the more active tissues such as liver than to the inactive tissues like muscle.

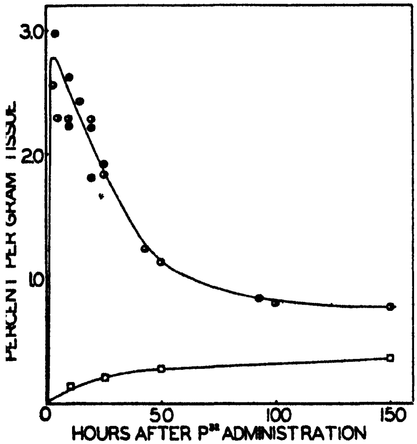


Fig. 1

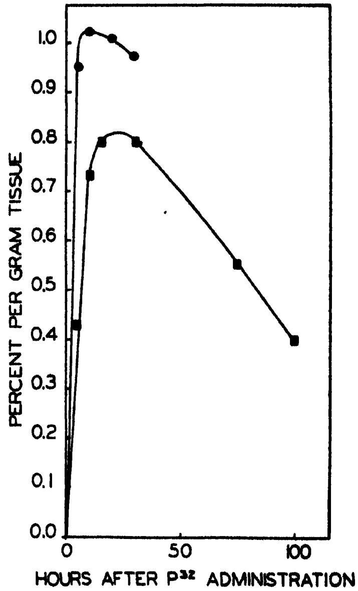


Fig. 2

Fig. 1. The labeled phospholipid content per gm. of liver and muscle removed from *normal* mice. The ordinates represent the per cent of administered phosphorus found as phospholipid per gm. of tissue. For the meaning of the symbols see Table I. Each point is the mean of four separate determinations on the tissues of four mice.

Fig. 2. The labeled phospholipid content per gm. of mammary carcinoma. The ordinates have the same meaning as for Fig. 1. For the meaning of the symbols see Table I. Each point is the mean of four separate determinations on tumors removed from four mice.

The curves obtained for the 9 and 28 day-old tumors were not alike; they differed most strikingly in the maximum phospholipid values found soon after P^{32} injections. The younger tumors had an average weight of 0.23 gm. (Table I) and were still actively growing, whereas the 28 day-old adenocarcinomas weighed 1.7 gm. after all extraneous or necrotic tissue had been removed and had reached a stage in their development where growth was very slow. This raises the question whether phospholipid metabolism of a tumor changes with its stage of development. Although it is recognized that an answer to this question must await further investigation, it should be noted here that the condition of the animal rather than the age of the tumor itself may be the factor determining the height of the curve, since toxic effects of this particular tumor may influence the rate of phospholipid turnover by various tissues. The animals in which the 28 day-old tumors were present were in much poorer condition than mice containing those 9 days old. Indeed, the old tumors had already reached the point of exerting lethal effects.

Lymphoma

The term lymphoma refers to a lymphoid neoplasm which, when transplanted subcutaneously, results in a localized tumor that later spreads and produces generalized disease with leucemia (10). Takes are uniformly successful and lead to death of the animal in 4 to 5 weeks after inoculation.

The phospholipid turnover of this neoplasm is shown in Fig. 3. These tumors were 14, 18, and 24 days old when the P^{32} was injected. They show a less active phospholipid metabolism than mammary carcinoma. At the height of the curve, each gm. of tumor contained about 0.3 per cent of the injected labeled phosphorus in the form of phospholipid, an amount that is one-half or one-third the maximum amount found in mammary tumors. The sharp rise and fall that characterized the more active tissues are also retained by this lymphoid neoplasm; the maximum was attained about 19 to 20 hours (Curves A, B, and C in Fig. 3) after the phosphorus injection and was followed by a sharp decline in the content of labeled phospholipid.

A number of these mice received a *second* injection of radioactive phosphorus when the tumors were 18 days old, and it is interesting to note that the sharp rise and fall in labeled phospholipid content first observed were roughly duplicated. The growth curve of this particular tumor is shown by the broken line in Fig. 3. Since Curve A, obtained at a time when rapid growth was occurring, resembled Curve B, which was obtained when growth had already attained a plateau, it would seem that the characteristic phospholipid activity of the lymphoma is not related to its particular rate of growth.

Curve C was derived from 24 day-old tumors in mice that had received a single injection of labeled phosphorus. The similarity in Curves A and C again shows that, so far as this neoplasm is concerned, age has no effect on its phospholipid turnover despite the fact that the older tumors were close to the time when they were exerting their lethal effects upon the mice.

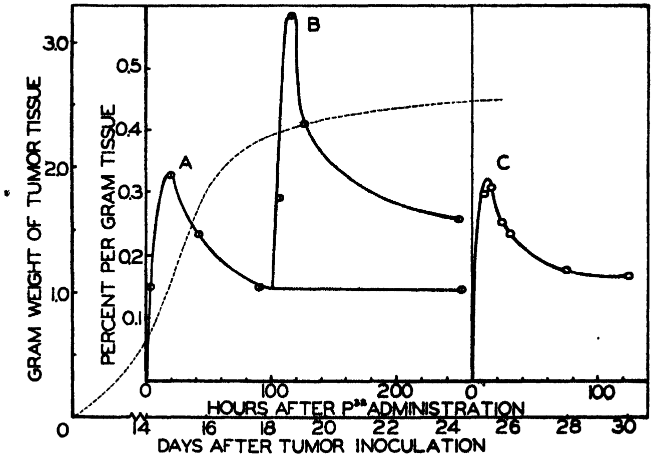


FIG. 3. The labeled phospholipid content per gm. of lymphoma. The ordinates have the same meaning as for Fig. 1. For the meaning of the symbols see Table I. Each point is the mean of three or four separate determinations on tumors removed from the same number of mice. The broken line is the growth curve for this tumor.

Lymphosarcoma

This is a transplantable tumor that does not produce a generalized disease but remains entirely localized.[2] It spreads quickly by continuity and grows to a large size locally. Histologically, it consists of round, closely packed cells which resemble the lymphoma.

---

[2] This tumor was induced by Dr. W. U. Gardner after prolonged folliculin

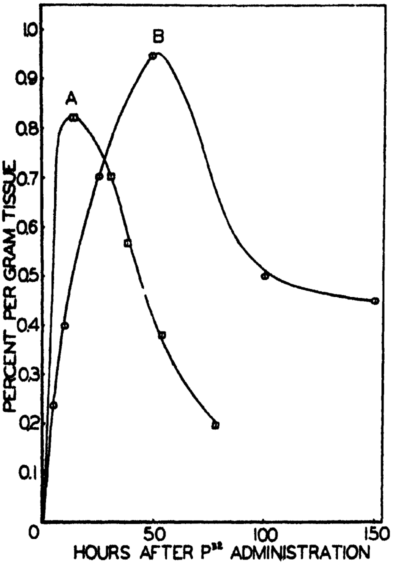


FIG. 4

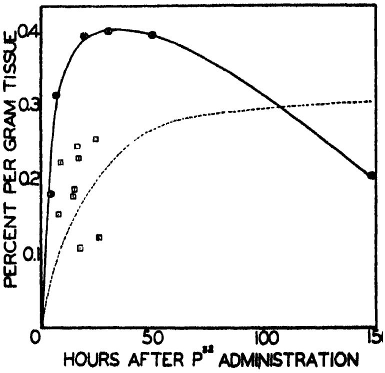


FIG. 5

FIG. 4. The labeled phospholipid content per gm. of lymphosarcoma. The ordinates have the same meaning as for Fig. 1. For the meaning of the symbols see Table I. Each point is the mean of two (Curve A) or four (Curve B) separate determinations of tumors removed from the same number of mice.

FIG. 5. The labeled phospholipid content per gm. of Sarcoma 180. The ordinates have the same meaning as for Fig. 1. Each point is the mean of three or four separate determinations of tumors removed from the same number of mice. The broken line is a duplicate of the muscle curve shown in Fig. 1. ● represents the data obtained on tumors in which no visible signs of regression could be detected (see Table I also); □, tumors in which spontaneous regression had occurred.

The data recorded in Figs. 1 to 3 were obtained from experiments in which the animals received the labeled phosphorus at

---

injections. We are indebted to him for the transplant used in this investigation.

the same time; namely, the zero time interval. The tumors removed at the end of the experiments were therefore older than those removed at earlier intervals, and the increase in age of a tumor during the period of observation is shown by the hours after P^{32} administration. Curve A (Fig. 4), showing the phospholipid activity of the 11 day-old lymphosarcoma, was obtained by this method of phosphorus injection. The mice shown in Curve B were not so treated. In this case the administration of the phosphorus was staggered so that the tumors shown in Curve B were all of the same age, namely 21 days, at the time they were excised. But it should be noted that, while all tumors shown in Curve B were of uniform age, the labeled phosphorus was injected at different stages in the development of the tumor.

In its general aspects, the phospholipid metabolism of lymphosarcoma greatly resembles that of mammary carcinoma. At the height of Curve B about 0.95 per cent of the injected labeled phosphorus was deposited as phospholipid per gm. of tumor tissue. It was noted above that the maximum amount of the injected labeled phosphorus incorporated into phospholipid by mammary carcinoma was about 1 per cent. Identical curves for phospholipid activity were not obtained for 11 and 21 day-old lymphosarcomas. Maximum activity was found at about 12 hours after the P^{32} injection in the younger tumor; the older one showed a slower rise, and the peak of the curve was not attained until 50 hours after the phosphorus administration. Rapid growth in this tumor begins at about 11 days after inoculation and the size of the tumor reaches a maximum at about 21 days.

Sarcoma 180

This transplantable tumor has been extensively used in experimental work. It is characterized by rapidity of growth and an absence of metastases, but it differs from the tumors recorded above in that it shows occasional spontaneous regressions.

The curve shown in Fig. 5 represents the phospholipid activity of 15 day-old tumors in which no visible signs of regression could be detected. The time of administration of the radioactive phosphorus was staggered so that all tumors were 15 days old at the time they were removed for analysis. In regard to the maximum values attained this tumor more closely resembles lymphoma than either mammary carcinoma or lymphosarcoma. At the height of the curve, 0.4 per cent of the injected phosphorus was found as phospholipid per gm. of tumor. While showing the rapid rise found in all three tumors recorded above, it differed from these in the rate at which labeled phospholipid left after the maximum amount had been deposited. The maximum amount of labeled phospholipid was retained for at least 50 hours, and even after that its removal occurred slowly. It still differed considerably from muscle, however, for the latter was gaining labeled phospholipid at a time when Sarcoma 180 was losing it.

In a number of mice, tumors in which spontaneous regressions had occurred were removed and analyzed for labeled phospholipid. The values obtained are recorded in Fig. 5. It is apparent that spontaneous regression in Sarcoma 180 is associated with a definite lowering in its phospholipid activity.

Phospholipid Turnover of Liver in Tumor-Bearing Mice

The phospholipid activity of the livers of tumor-bearing mice is shown in Fig. 6. Two groups of mice with mammary carcinoma were examined; in one the tumors were 9 days old, in the other 21 days. Normal activity was observed in the livers removed from mice containing the 9 day-old tumors. It should be noted that at this stage in their development the tumors are small, weighing between 0.04 and 0.2 gm. The livers obtained from mice bearing 28 day-old mammary carcinoma, 21 day-old lymphosarcoma, and 15 day-old Sarcoma 180 showed a lowered activity in the early period after the P^{32} administration. This period corresponds to the interval during which the most rapid phospholipid turnover is found in normal livers. Between the intervals of 50 and 100 hours after the P^{32} administration, the content of labeled phospholipid in the liver of mice bearing mammary carcinoma, lymphosarcoma, and Sarcoma 180 corresponded closely with the values observed in normal mice. At this interval the rapid rise and fall in phospholipid turnover had already been passed; the curve for the phospholipid content of the liver between these two times is nearly a straight line, representing a value approximating 1 per cent of labeled phospholipid per gm. of liver.

The livers obtained from mice containing 24 day-old lymphoma showed a depressed phospholipid activity per gm. of tissue before as well as after the 50 hour interval following the P^{32} administration. It is this tumor that gives rise to generalized lymphomatosis in the later periods after inoculation. The lowered phospholipid activity per gm. of liver is probably the result of an enlargement of the liver (see Table I) by lymphoid infiltration.

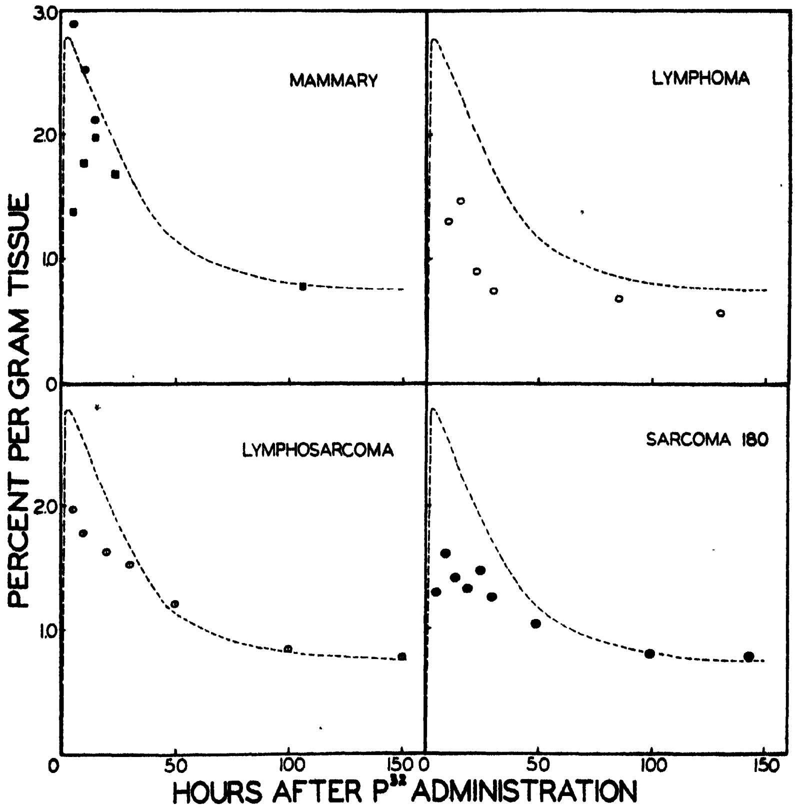


FIG. 6. The labeled phospholipid content per gm. of liver removed from tumor-bearing mice. The ordinates have the same meaning as for Fig. 1. Each symbol is the mean of three or four separate analyses on whole livers obtained from *tumor-bearing mice* shown by corresponding symbols in Table I. The broken line is a duplicate of the liver curve for *normal* mice shown in Fig. 1, and is presented here for comparison with the livers removed from tumor-bearing mice.

Each gm. of hepatic tissue is thus diluted with a tumor which, as shown above, has a much lower phospholipid activity than the tissue it is invading. Other factors undoubtedly play a part in depressing the phospholipid activity of the liver in tumor-bearing mice; this is shown in the experiments with tumors that did not metastasize, although the mechanism involved here is a subject for further investigation.

DISCUSSION

The phospholipid turnover of four tumors transplanted into mice was determined with the aid of radioactive phosphorus. They differed in such respects as cell type and rate of growth. Two of these produced generalized disease or metastases in distant parts, whereas two others remained entirely localized at the place of inoculation. Moreover, one of the tumors studied showed regressive change spontaneously. *A uniform phospholipid activity was not found in the types of tumors examined,* an observation worthy of note since general views regarding the metabolism of tumors have not uncommonly been drawn from investigations in which a single type was used. Differences in the extent of the phospholipid turnover in the four types of tumor employed were as great as, or greater than, those found among normal tissues. Thus the activity of lymphoma was only about one-third of that found in mammary carcinoma or lymphosarcoma.

The highest rate of phospholipid turnover was found in the mammary carcinoma and in the lymphosarcoma, and judging by the maximum amounts of labeled phospholipid deposited after the injection of radioactive phosphorus, the phospholipid activity of these two tumors was at least twice as great as that observed in Sarcoma 180 and lymphoma. *Cell type is apparently not a decisive factor determining the extent of phospholipid activity,* for similar activities were found in tumors with such widely differing cell patterns as mammary carcinoma, which is epithelial, and lymphosarcoma, which is mesothelial in origin. Moreover, the lymphosarcoma was more than twice as active as the lymphoma despite the fact that the cells that make up these two lymphoid tumors bear a great resemblance to each other. It would therefore seem that each tumor pursues its own particular phospholipid metabolism and that this is independent of the cell type involved.

The general characteristics of phospholipid metabolism in tumors may now be considered. These are particularly well brought out by comparing the phospholipid turnover of the four types of tumors studied here with the phospholipid activity found in (1) muscle of normal mice and (2) liver of normal and tumor-bearing mice. Although in none of the tumors examined did the height of activity approach the maximum activity of the liver, the metabolism of these tumors nevertheless bore a greater resemblance to the more active of the normal tissues such as liver, kidney, and small intestine than to the less active tissues such as muscle and brain. The characteristic feature of the former, *i.e.* the rapid rise in content of labeled phospholipid very early after the administration of labeled phosphorus, was particularly well shown by all four tumors. The highest activity observed among them all was approximately one-third of that found in livers of normal mice, whereas the lowest was about one-tenth. Even in cases in which the presence of the tumor had a depressing effect upon the phospholipid metabolism of the liver, the phospholipid turnover of the latter was still much greater than that of the tumor. It is of particular importance to note that the phospholipid metabolism of these tumors bears no resemblance to that of muscle, and this conclusion is strikingly borne out by the finding that muscle is still increasing its content of labeled phospholipid at a time when all four neoplasms were either rapidly or slowly losing it.

SUMMARY

The phospholipid turnover of four tumors was investigated with the aid of radioactive phosphorus. These tumors included a mammary carcinoma, a lymphoma, a lymphosarcoma, and Sarcoma 180.

1. The phospholipid metabolism of these four tumors was not uniform in either height or type of activity.

2. Cell type is not the factor determining the level of the phospholipid activity in a tumor.

3. Judging by the speed with which labeled phospholipid is deposited after the injection of P^{32}, the phospholipid turnover of tumors bears a greater resemblance to that of the more active tissues such as liver, kidney, and intestine than to that of the less active tissues like muscle or brain.

4. The phospholipid content of the liver is depressed when large tumors are present in mice, particularly during the early period following the P^{32} administration. The phospholipid activity of the liver in normal as well as in tumor-bearing mice is several times greater than the highest activity observed in tumors.

The assistance furnished by the Works Progress Administration (Official Project No. 665-08-3-30, Unit A6) is gratefully acknowledged. Our best thanks are also due to Mrs. Virginia Condit Eggleston for assistance in the preparation of the tumor-bearing animals used in this investigation.

BIBLIOGRAPHY

1. Haven, F. L., *J. Biol. Chem.*, **118**, 111 (1937).
2. Perlman, I., Ruben, S., and Chaikoff, I. L., *J. Biol. Chem.*, **122**, 169 (1937-38).
3. Changus, G. W., Chaikoff, I. L., and Ruben, S., *J. Biol. Chem.*, **126**, 493 (1938).
4. Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **127**, 211 (1939).
5. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **108**, 201 (1935).
6. Entenman, C., Ruben, S., Perlman, I., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, **124**, 795 (1938).
7. Strong, L. C., *J. Hered.*, **27**, 21 (1936).
8. Gardner, W. U., Smith, G. M., Allen, E., and Strong, L. C., *Arch. Path.*, **21**, 265 (1936).
9. Lawrence, J. H., Horn, R., and Strong, L. C., *Yale J. Biol. and Med.*, **10**, 145 (1937).
10. Lawrence, J. H., and Gardner, W. U., *Am. J. Cancer*, **33**, 112 (1938).

DEPOSITION AND UTILIZATION OF FATTY ACIDS

I. FAT SYNTHESIS FROM HIGH CARBOHYDRATE AND HIGH PROTEIN DIETS IN FASTED RATS*

By HERBERT E. LONGENECKER†

(*From the Department of Biochemistry, Queen's University, Kingston, Canada*)

(Received for publication, February 27, 1939)

The study of many fats in recent years has led to considerable systematic information regarding their fatty acid composition. Our knowledge has been qualitatively extended into both the animal and vegetable kingdoms to such a degree that at present the outstanding differences between families and species are clearly recognized. Gradual changes paralleling the position in the scale of evolutionary development have been suggested (1).

The mass of data obtained in these investigations has indicated the general characteristics for the depot fat composition of several species. At the same time, the possibilities of variance in the same species have been forcibly demonstrated. Of those factors whose effect on the composition of animal depot fat has been studied—diet, age, sex, activity, temperature, site of deposition—diet seems to be the most significant. The literature on this subject has been reviewed recently by Anderson and Williams (2).

In earlier work on animal fats, fatty acid compositions were customarily inferred on the basis of a single analytical value, the iodine number. Changes in this number were taken to indicate differing proportions of fatty acids. Thus, carbohydrate or protein was shown to produce a body fat in the rat approaching an iodine value of 60 to 65 (3, 4); the marked effect of food fat upon body fat was summarized by an impressive parallel series of iodine numbers (3); and the "hardening" influence of high carbohydrate, low fat diets upon soft fats (of high iodine value) was demonstrated by lowered iodine values for body fat of hogs (5) and rats (3, 6).

---

* Part of these data was presented before the meeting of the American Oil Chemists' Society at Chicago, October 6 to 7, 1938.

† National Research Council Fellow in the Natural Sciences.

It is unfortunate that these effects were not demonstrated in terms of fatty acid compositions rather than iodine numbers. It is not implied that incorrect conclusions were reached in these and other similar cases. It is essential, however, to remember that the iodine absorption value is only a mean figure and that a single value may represent several possible combinations of fatty acids, involving both degree of unsaturation and size of the molecule.

Mendel and Anderson (7) commented on their own work as follows: "Close similarities between food fat and body fat by no means always develop." They also wrote about such discrepancies in the results when coconut oil was fed: "Whether this outcome is secured by actual desaturation in the organism or by a preferential catabolism of the more saturated fatty acids, resulting in a less saturated residuum in the fat depots, remains to be ascertained by detailed comparisons of the different fatty acids involved."

It is indeed surprising that few such comparisons have been made. The specific effects of the *kind* of fat in the diet on the *kind* of fat deposited are generally recognized (6, 8, 9). Little is known of the effect of the amount of fat in the diet upon the composition of the body fat (*cf.* Spadola and Ellis (6)). Only the general effects of carbohydrate and protein on depot fat mentioned above have been indicated by previous investigators (3, 4, 10, 11).

The present study is an attempt to clarify some of the "unknowns" in fat metabolism under discussion here by a detailed examination of the fatty acids deposited in rats after they have been fed diets of known compositions. Specifically, one object of the work was to make a comparative study of the fatty acids synthesized from diets very low in fat, and high in either carbohydrate (sucrose or corn-starch) or protein (casein).

Adult animals were used in most cases and the synthesis of fat was measured after a preliminary period of inanition which served to reduce their preformed fat stores.[1] The question naturally arose as to whether one could assume that the composition of the depot fat remained the same during inanition or whether, as it was being used to supply the demands of the organism, there was a preferential removal of certain of the deposited fatty acids. Another objective in these studies, then, was to investigate the possibility of differential utilization of fatty acids.

---

[1] The term *preformed fat* appears several times in this communication. By it is meant the total body fat at an indicated time; it should not be confused with dietary fat. *Body fat* is used here synonymously with *depot fat*, *reserve fat*, or *storage fat* to indicate the total acetone-soluble

EXPERIMENTAL

General Plan of Study—Male albino rats were grown on a stock diet of Purina Dog Chow to about 275 gm.; females were raised similarly to about 190 gm. The body fat deposited in rats on this widely used ration was determined in two groups of animals. Those of Group 1 were all males; those of Group 10, all females.

In several other groups, a period of inanition was used to reduce in amount this preformed body fat in order to study the quantity and nature of fat synthesized from each carbohydrate and protein. In the case of Group 4, all males, inanition was continued until 24 per cent of their original weight was lost. This lost weight was regained in 27 days on Diet 300 in which 84 per cent of the calories was contributed by sucrose and 16 by casein, with 4 parts per hundred of salts (12). The diet was supplemented daily by 500 mg. of dried yeast and 1 drop of diluted haliver oil (1:50 in mineral oil). Male animals, Group 6, were raised to about 240 gm. on the Dog Chow and then fed Diet 300 for the same length of time, 27 days, as was required by the fasted animals, Group 4, to regain their original weight on the same diet. The rats of Group 5, all males, were fasted to a 27 per cent loss in weight and then fed a very high protein ration, casein 96 parts and salts (12) 4 parts, supplemented daily by 500 mg. of dried yeast and 1 drop of the diluted haliver oil. A group of females, Group 20, was again fed the original ration, Purina Dog Chow, after a fasting period during which the animals lost 30 per cent of their body weight.

In order to establish the residual amount of preformed body fat in fasted animals, and to determine whether it differed in nature from the original, Groups 2 and 3, males, were fasted until they lost in weight 28 and 17 per cent, respectively.

---

glycerides or "neutral" fat (as distinct from phospholipids, cerebrosides, etc.) in the entire animal exclusive of the organs and gastrointestinal tract.

Groups 18 and 19 were raised from weaning (at 40 gm.) on a low fat, high carbohydrate diet, No. 301, which resembled Diet 300 mentioned above in every way except that corn-starch replaced sucrose. Preformed fat stores, however small, were depleted by a short period of inanition. At about 165 gm., the animals in Group 18 were sacrificed and the ones in Group 19 were fasted to a 22 per cent loss in body weight. Again, it was the purpose of this experiment to define in at least a semiquantitative fashion the fatty acid formation and deposition on a diet very low in fat (0.2 to 0.3 per cent) and also the nature of the remaining fatty reserve tissue after inanition.

TABLE I

Average Animal Weights and Treatment

Group No.	No. of animals	Initial weight	After inanition	Loss in weight	After refeeding period
		gm.	*gm.*	*per cent*	*gm.*
1	8	251			
2	9	255	182	28	
3	10	268	222	17	
4	10	295	224	24	306
5	8	269	195	27	267
6	9	238			282
10	8	225			
20	7	185	130	30	177
18	4	67	56	16	164
19	8	75	64	14	166*

* These animals were fasted to an average body weight of 129 gm. (22 per cent loss in weight).

The average weights of animals in each group at the beginning of an experiment, after the preliminary inanition period, and at the end of the refeeding period are shown in Table I.

Method of Obtaining Depot Lipids or Reserve "Fat"—It was desirable to obtain essentially blood-free carcasses and for this reason the animals were decapitated. The organs and the complete gastrointestinal tract were removed, care being taken to preserve with the carcass as much of the mesentery and renal depots as possible. The carcasses and hides were ground in a meat chopper and placed in 3 to 4 liters of redistilled acetone immediately. For these analyses, the carcasses from each group of animals were pooled. Acetone was used because it is an effective dehydrating agent as well as lipid solvent. Total extraction of lipid material was readily accomplished by simply refluxing the wet tissue in three successive 3 liter portions of redistilled acetone followed by two 2 to 3 liter portions of peroxide-free ether.[2] Most of the water present originally in the tissues and a considerable fraction of the total lipids were found in the first acetone filtrate with decreasing amounts of water in the second and third acetone extracts, respectively. In no case did the ether finally remove more than 2 gm. of lipids. (That complete extraction of the tissue was obtained was shown by alkaline digestion of the residues and extraction of only traces (0.1 to 0.2 gm.) of ether-soluble material after acidification.)

The acetone and ether extracts were concentrated under nitrogen or carbon dioxide to prevent oxidative changes. An ether solution of the combined extracts was then washed with distilled water, the washings being reextracted twice with ether. After the extracts were dried with anhydrous sodium sulfate or anhydrous calcium sulfate, the ether solution of the total lipids was diluted to a definite volume and aliquots were taken to determine the weight of total material extracted.

The hot acetone extraction was in itself sufficient for the removal of most of the phospholipids from the carcasses. Their extraction along with neutral glycerides and other lipids was facilitated in the above procedure by the dilution of the acetone, owing to dehydration of the tissues. It was desirable to effect a rigorous separation of phospholipids from "neutral" glycerides which represent the depots. This division was accomplished in the usual fashion after concentration of the ether solution of the total extract (under N_2 or CO_2). The total lipids were poured into 1 to 2 liters of acetone (5 to 10 cc. of acetone per gm. of total extract). A precipitate which formed within a few minutes at room temperature was centrifuged and the separation of the phospholipids completed by repeated additions of small amounts of $MgCl_2$ in alcoholic solution to the supernatant liquid until no more precipitate was obtained. The clear supernatant acetone solution was made up to a definite volume and aliquots indicated the amounts of "neutral" glycerides (plus some unsaponifiable matter) that had been obtained. Data indicating the amounts and general nature of the acetone-soluble lipids may be found in Table II.

---

[2] These solvents are readily freed of the bulk of interfering contaminating products by distillation over NaOH.

During the separation of phospholipids in the cases of Groups 4, 5, 6, 18, and 19, precautions mentioned previously (9) had to be taken to avoid the crystallization of "neutral" glycerides, which were in all likelihood disaturated glycerides. The rat resembles other animals which have small amounts of stearic acid present in their depot fats in having correspondingly small amounts of trisaturated glycerides (14).

TABLE II

General Characteristics of Total Acetone-Soluble Lipids in Rats

The animals in Groups 10 and 20 were females; all others were males.

Group No.	Diet and treatment	Acetone-soluble lipid			Lead salt separation of mixed fatty acids			
		Per cent of total body weight	Saponification equivalent	Iodine No.*	Solid acids		Liquid acids	
					gm.	*per cent*	*gm.*	*per cent*
1	Dog Chow	5.0	294.3	78.3	20.15	25.2	59.67	74.8
2	" " then fasted, 28% loss	1.1	290.6	77.2	2.14	17.8	9.85	82.2
3	Dog Chow, then fasted, 17% loss	3.7	292.3	77.5	15.52	23.2	51.35	76.8
4	High carbohydrate Diet 300 (after a fast, 24% loss)	10.2	285.4	62.5	26.75	29.4	64.45	70.6
5	High protein Diet 200 (after a fast, 27% loss)	10.5	284.9	60.3	28.45	31.7	61.43	68.3
6	Dog Chow to Diet 300	10.7	288.1	68.5	33.01	35.0	61.23	65.0
10	" "	9.8	292.5	81.1	25.43	26.0	72.51	74.0
20	" " (after a fast, 30% loss)	5.3	290.5	76.4	14.75	30.4	33.85	69.6
18	High carbohydrate Diet 301	8.4	286.2	56.2	14.91	31.1	33.11	68.9
19	" " then fasted, 22% loss	3.9	286.4	56.2	12.39	34.6	23.40	65.4

* The Rosenmund-Kuhnhenn (13) method was used throughout.

Analysis of Acetone-Soluble Lipids—The acetone-soluble lipids were examined in detail for fatty acid composition. The fatty acids obtained after saponification in aldehyde-free alcohol were resolved into two groups by the modified Twitchell lead salt procedure (*cf.* (14)). Nearly quantitative recovery of the acids from both the soluble ("liquids") and insoluble ("solids") lead salts was effected after their decomposition with an excess of hydrochloric acid. Each group of acids was esterified with dry, recently distilled methyl alcohol which was aldehyde-free. 10 to 20 times the theoretical amount of methyl alcohol containing 5 per cent sulfuric acid as a catalyst was used. Any unchanged fatty acid was removed by washing an ether solution of the esters first with a 5 per cent solution of potassium carbonate and then with distilled water until the washings were carbonate-free. Practically complete (about 98 per cent) esterification was regularly obtained.

TABLE III

Composition of Rat Depot Fats

All values are given in molar percentages. For a brief description of the diet and treatment of each group, *cf.* Table II.

Acids	Group No.									
	1	2	3	4	5	6	10	20	18	19
Myristic	1.6	1.8	2.0	3.1	2.8	2.7	1.8	3.1	2.9	4.0
Palmitic	21.6	21.8	21.6	26.7	29.7	28.2	24.4	23.0	30.4	28.8
Stearic	3.6	3.7	4.6	3.6	3.8	4.3	3.6	7.2	4.9	4.6
Arachidic	2.0	1.2	1.6	0.4	1.1	1.2	0.8	0.7		0.3
Tetradecenoic		0.3	0.8	0.9	1.0	1.2	0.3	1.7	1.3	1.9
Hexadecenoic	4.1	4.8	5.7	15.6	15.6	12.2	4.8	10.0	13.1	7.2
Oleic	51.9	51.3	43.4	47.2	43.8	42.0	44.3	39.9	46.0	51.0
Octadecadienoic	13.0	13.5	19.2	2.2	2.0	8.0	18.6	13.3	1.4	2.2
Arachidonic	2.2	1.6	1.1	0.3	0.2	0.2	1.4	1.1		
Totals										
C_{14}	1.6	2.1	2.8	4.0	3.8	3.9	2.1	4.8	4.2	5.9
C_{16}	25.7	26.6	27.3	42.3	45.3	40.4	29.2	33.0	43.5	36.0
C_{18}	68.5	68.5	67.2	53.0	49.6	54.3	66.5	60.4	52.3	57.8
C_{20}	4.2	2.8	2.7	0.7	1.3	1.4	2.2	1.8	0.0	0.3
% saturated	28.8	28.5	29.8	33.9	37.4	36.4	30.6	34.0	38.2	37.7
% unsaturated	71.2	71.5	70.2	66.2	62.6	63.6	69.4	66.0	61.8	62.3

All solvent distillations from soaps, fatty acids, and esters were carried out in a stream of N_2 or CO_2, preferably the former because of the lesser effect on subsequent molecular weight determinations due either to absorption or reaction.

A 1.5 cm. distillation column, No. 15-Ag, packed for 60 cm. with 3 mm. single turn, glass helices was used for the separation of all ester mixtures with results as typified in Table III. The column (15) was electrically heated and equipped for total condensation and partial take-off of the distillate. Its efficient operation in the separation of known fatty acid ester mixtures under low pressures has led to its use in analyses of such mixtures as are obtained from natural fats (*cf.* Hilditch and Jasperson (16)).

Use of an efficient fractionation apparatus is imperative in these studies for the avoidance of distillate fractions in which one might have C_{14}, C_{16}, and C_{18} esters, and for the maximum resolution of adjacent groups. Calculation of the composition of a mixture of adjacent groups, *e.g.* C_{14} and C_{16} or C_{16} and C_{18}, is quite readily accomplished, however, by standard methods (14, 9, 6) and these must be resorted to for the inevitable mixtures which result. The complete results of all the analyses (*cf.* Table III) are given in molar percentages. All data pertaining to distillations and calculations from analytical values of ester fractions have been omitted to conserve space.

DISCUSSION

Synthesis of Fatty Acids from Carbohydrate and Protein—The synthesis of fat from both carbohydrate and protein in adult animals is clearly demonstrated in Groups 4 and 5. Fasting of adult male rats lowered the total reserves of body fat from 5.0 per cent of the total body weight (Group 1) to 1.1 per cent (Group 2). The latter figure may fairly be taken as an index of the amount of preformed fat in Groups 4 and 5 at the time the high carbohydrate and high protein rations were fed. It is then possible to calculate the amount of fat laid down as a direct result of feeding these rations. In the case of Group 4, the fat deposited amounted to 35.1 per cent of the total weight regained. The animals in Group 5 similarly deposited 36.1 per cent of the total gain as neutral fat. These percentages represent 20 to 30 times the maximum amount of lipids present in the total ration consumed during the refeeding period. Thus more than 95 per cent of the lipids deposited during the refeeding period (about 4 weeks) has resulted from the non-lipid energy-yielding constituents, carbohydrate and protein. It may be observed that practically the same fat synthesis was obtained from protein as from carbohydrate.

Synthesis of fat from carbohydrate is an old observation (17). Likewise, the production of sugar from protein was demonstrated long ago (18). It would seem to follow from these facts that protein should be convertible into fat. The actual demonstration of such conversion, however, has met many experimental difficulties. Mitchell and Hamilton (19) after thoroughly considering the earlier literature reached the following judgment: "a clear cut demonstration of the production of fat from protein in the higher animals has not yet been reported."

The final fatty acid compositions obtained for Groups 4 and 5 represent a small amount of preformed fat, "synthetic" fat derived from carbohydrate and protein, and possibly some lipid deposited directly from the small amount in the ration. The composition of the preformed fat residing in the tissues after the preliminary inanition may be assumed to approximate the original (*cf.* later discussion) and that present in the fat of the diet may be determined in the usual fashion. Using these figures one can readily obtain a composition for the synthetic fat. A close approximation which is not significantly different from that for the animals of Group 18, raised on a low fat-high carbohydrate diet from weaning, may be made by considering only the residual preformed fat and the final extract obtained.

The nature of the fatty acid mixture which was formed in these cases from protein and carbohydrate is indeed interesting. It is evident (*cf.* Table III) that high amounts of C_{16} acids (40 to 45 per cent) are present in such synthetic fat. Particularly striking is the high amount of the mono unsaturated C_{16} acid, sometimes referred to as palmitoleic acid but preferably called hexadecenoic acid to avoid confusion (*cf.* (16)). In Groups 4 and 5, the values for this acid reached the extraordinary figure of 15.6 per cent of the total mixed acids, and in Group 18, 13.1 per cent was attained. The animals changed from a Dog Chow ration directly to the high carbohydrate diet increased their deposits of palmitoleic acid from 4.1 (Group 1) to 12.2 per cent (Group 6).

It is somewhat puzzling that this increase was so marked. The Dog Chow itself is high in digestible carbohydrate and protein. Analyses of the ration show an average lipid content of about 5 per cent, whereas the diets used here were much lower in fat. It is possible that in some unknown manner this may be the determining factor. Further experiments are in progress to test the point. Spadola and Ellis (6) obtained results very similar to the ones listed above. With a diet very similar to the one used here for Group 18, they found 14 per cent of the mixed acids as hexadecenoic acid. Replacement of 8 per cent of the dextrin in their diet by fat lowered the amount of this acid, and the decrease appeared to be related to the amount of linoleic acid in the ingested fat.

Hexadecenoic (Palmitoleic) Acid in Animal Depot Fats—Hexadecenoic acid has for some years been recognized as a well characterized constituent of whale (20) and fish (21) oils. Its probable occurrence in the body fat of white rats was suggested by Banks, Hilditch, and Jones (22) and soon confirmed (23, 24, 6, 8, 9). That it is not a peculiar acid found only in rare species is further indicated by the discovery of it in the depot fats of a wider variety of animals; *e.g.*, amphibians and reptiles (23, 25, 26), birds (27), and such higher land animals as the cow (14, 28) and pig (29). More than passing reference need not be made here to its generally recognized occurrence in liver (*cf.* (29)) and milk fats (24, 30).

This previous evidence of its general occurrence in animal fats together with the data presented here showing the marked effect of the type of diet on the relative amount of hexadecenoic acid makes it desirable to investigate other species for similar effects. It is clear that considerable quantities of hexadecenoic acid have been synthesized from carbohydrate and protein in addition to the well recognized oleic acid. It is, perhaps, premature to do more than call attention to a possible relation between carbohydrate and protein metabolism and the formation of this C_{16} unsaturated acid.

In most previous writings on the occurrence of hexadecenoic acid in animal depot fats it has been considered a minor constituent except in the marine fauna. Certainly the cases reported here make it necessary to consider its inclusion among the major component acids which are variable, as, for example, linoleic (octadecadienoic) acid whose presence depends upon dietary habits of the animal.

Utilization of Fatty Acids during Inanition—It might be expected, *a priori*, that unsaturated fatty acids being more susceptible to oxidation *in vitro* than saturated fatty acids would be first utilized *in vivo* as the organism uses its stores of fat to meet the energy requirements imposed by inanition. One might reason with equal force that the utilization of the fat reserves for energy would result (by desaturation) in the production of more highly unsaturated molecules prior to their ultimate complete oxidation. The first idea is more readily subjected to experimental test than the second, for if any one particular fatty acid or types of fatty acids are burned in preference to the others, then the molar distribution of the acids before and after utilization of depot fats should clearly reveal the fact. The second case, however, might easily be complicated by a chain reaction completely removing any more unsaturated acids by immediate complete oxidation, which would have the effect of nullifying an opportunity for detecting them by this method of final fatty acid compositions. Such chain reactions would not present a complication in the former case.

In examining the fatty acid compositions of depot fats from the groups of fasted rats (Groups 2, 3, and 19), one may find an indication of the processes that are operative. Some changes in the make-up of the acid mixtures is apparent, but it is equally obvious that these cannot yet be taken as evidence for either of the above hypotheses. Thus the composition of Group 2, fasted to a 28 per cent loss in body weight, is scarcely altered from that of Group 1 which was comparable in all respects (age, weight, diet, sex) except for the period of inanition. The animals of Group 19 were likewise comparable with those of Group 18. They show some evidence of change, with definitely lower amounts of palmitic and hexadecenoic acids and higher amounts of the C_{18} unsaturated acids.[3] This change was not, however, indicated by the iodine numbers of the acetone-soluble lipids obtained (*cf.* Groups 18 and 19, Table II), which result was very similar to that obtained by Reed, Yamaguchi, Anderson, and Mendel (11) on fasting their starch-fed group of animals.

---

[3] The case of Group 3 might be cited also to indicate an increase in C_{18} unsaturated acids were it not for the fact that these animals were somewhat older than those of Groups 1 and 2. Some evidence of increasingly higher amounts of octadecadienoic acid in older animals was found during this work. The evidence is being checked further at the present time.

Some Typical Features of Rat Depot Fat—These analyses extend several previous observations on the nature of the rat's reserve fat. Its composition appears to be regulated by the amount and type of fat present in the diet to an extent not previously understood. When fat is essentially lacking in the ingested food, the major component acids are oleic, palmitic, and hexadecenoic, the minor components being myristic and tetradecenoic, stearic and octadecadienoic, arachidic and arachidonic acids. With a 5 per cent fat ration containing octadecadienoic acid such as the Dog Chow used here, oleic, palmitic, and octadecadienoic acids are the major components, hexadecenoic acid being found among the minor components. The octadecadienoic acid is apparently directly deposited.

The threshold level for saturated acids which Barbour (31) considered 25 to 27 per cent was reinterpreted by Longenecker and Hilditch (9) to be more nearly "seven to ten units higher." This latter interpretation is substantiated in the present work. It is fully appreciated that exceptional conditions, *e.g.* feeding diets unusually high in saturated acids of low molecular weight (8), will raise this level appreciably. The tendency, however, is to restrict the saturated acids to 35 to 37 per cent of the total mixed acids.

Rat body fat is peculiarly characterized by its relatively low proportions of stearic acid (about 4 per cent). This acid is notably variable in amounts in different animals (*cf.* (14)). When it forms only 5 to 10 per cent of the total acids, glyceride structure studies of the entire fat have indicated an even distribution of the component fatty acids (22, 32).

SUMMARY

1. Fat deposition in rats was studied to determine the molecular distribution of fatty acids when the animals were under known dietary regimens. On a 5 per cent fat ration in which linoleic acid was present, the animals regularly laid down a reserve fat in which oleic, palmitic, and linoleic acids predominated.

2. Direct experimental evidence of fatty acid synthesis from both carbohydrate and protein was obtained. Adult, fasted rats, refed high carbohydrate or high protein diets, synthesized large quantities of fat. One-third of their total gain in weight during the refeeding period was deposited as fat. It was shown that at least 95 per cent of this fat was derived from the non-lipid constituents of the ration.

3. When depot fat was synthesized from carbohydrate or protein, the molar distribution of fatty acids was strikingly different from that when 5 per cent fat was present in the food. Acids of the C_{16} series totaled 40 to 45 per cent in the body fat derived from non-lipid dietary sources but only 25 to 30 per cent from the 5 per cent fat ration; whereas, the total C_{18} acids were 50 to 55 per cent and 67 to 68 per cent, respectively. Unusually large amounts of hexadecenoic acid (13.1 to 15.6 per cent) were present in the "synthetic" body fat. Small amounts (about 4 per cent) of the C_{16} unsaturated acid and directly deposited linoleic acid (about 13 per cent) in fat formed on the 5 per cent fat ration accounted for the differences.

4. Only slight changes in the proportions of the component fatty acids were observed when depot fats were utilized by fasting animals to 15 or 25 per cent loss in weight.

5. A tendency to restrict the saturated acids to about 36 per cent of the total acids was observed.

For his kind hospitality and friendly counsel, the author is deeply indebted to Dr. R. G. Sinclair. The suggestions of Dr. C. G. King in the preparation of the manuscript are gratefully acknowledged.

BIBLIOGRAPHY

1. Hilditch, T. P., and Lovern, J. A., *Nature*, **137**, 478 (1937).
2. Anderson, W. E., and Williams, H. H., *Physiol. Rev.*, **17**, 335 (1937).
3. Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **76**, 729 (1928).
4. Eckstein, H. C., *J. Biol. Chem.*, **81**, 621 (1929).
5. Ellis, N. R., and Isbell, H. S., *J. Biol. Chem.*, **69**, 219 (1926). Ellis, N. R., Rothwell, C. S., and Pool, W. O., *J. Biol. Chem.*, **92**, 385 (1931). Ellis, N. R., *U. S. Dept. Agric., Tech. Bull. 368* (1933).
6. Spadola, J. M., and Ellis, N. R., *J. Biol. Chem.*, **113**, 205 (1936).
7. Mendel, L. B., and Anderson, W. E., *Yale J. Biol. and Med.*, **3**, 107 (1930).

8. Channon, H. J., Jenkins, G. N., and Smith, J. A. B., *Biochem. J.*, **31**, 41 (1937).
9. Longenecker, H. E., and Hilditch, T. P., *Biochem. J.*, **32**, 784 (1938).
10. McAmis, A. J., Anderson, W. E., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, **26**, 516 (1929).
11. Reed, L. L., Yamaguchi, F., Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **87**, 147 (1930).
12. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).
13. Rosenmund, K. W., and Kuhnhenn, W., *Z. Untersuch. Nahrungs- u. Genussmittel*, **46**, 154 (1923).
14. Hilditch, T. P., and Longenecker, H. E., *Biochem. J.*, **31**, 1805 (1937).
15. Longenecker, H. E., *J. Soc. Chem. Ind.*, **56**, 199 T (1937).
16. Hilditch, T. P., and Jasperson, H., *J. Soc. Chem. Ind.*, **57**, 84 (1938).
17. Lawes, J. B., and Gilbert, J. H., *Brit. Assn. Adv. Sc., Rep.*, 323 (1852); *J. Roy. Agric. Soc. England*, **14**, 439 (1853).
18. Ringer, A., and Lusk, G., *Z. physiol. Chem.*, **56**, 106 (1910).
19. Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, American Chemical Society monograph series, New York, 321 (1929).
20. Armstrong, E. F., and Hilditch, T. P., *J. Soc. Chem. Ind.*, **44**, 180 T (1925).
21. Toyama, Y., *J. Soc. Chem. Ind., Japan*, **30**, 603 (1927).
22. Banks, A., Hilditch, T. P., and Jones, E. C., *Biochem. J.*, **27**, 1375 (1933).
23. Klenk, E., Ditt, F., and Diebold, W., *Z. physiol. Chem.*, **232**, 54 (1935).
24. Spadola, J. M., and Riemenschneider, R. W., *J. Biol. Chem.*, **121**, 787 (1937).
25. Klenk, E., *Z. physiol. Chem.*, **221**, 264 (1933).
26. Hilditch, T. P., and Paul, H., *Biochem. J.*, **31**, 227 (1937).
27. Hilditch, T. P., Jones, E. C., and Rhead, A. J., *Biochem. J.*, **28**, 786 (1934).
28. Hilditch, T. P., and Paul, S., *Biochem. J.*, **32**, 1775 (1938).
29. Hilditch, T. P., and Shoreland, F. B., *Biochem. J.*, **31**, 1499 (1937).
30. Hilditch, T. P., and Longenecker, H. E., *J. Biol. Chem.*, **122**, 497 (1938).
31. Barbour, A. D., *J. Biol. Chem.*, **106**, 281 (1934).
32. Hilditch, T. P., *Analyst*, **62**, 250 (1937).

EFFECT OF THYROID FEEDING AND THYROIDECTOMY ON THE OXIDATION OF AMINO ACIDS BY RAT KIDNEY AND LIVER*

By J. R. KLEIN

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, December 9, 1938)

It has been shown that the rate of oxidation of a mixture of α-naphthol and dimethyl-*p*-phenylenediamine by liver and kidney is decreased in hypothyroidism (1, 2). Thyroidectomized pups and lambs were used. The literature concerning the effect of hyperthyroidism and hypothyroidism upon the rate of oxidation of various substrates by other tissues is summarized by Gerard and McIntyre (3), McEachern (4), and Cohen and Gerard (5).

The present study was made in order to establish whether changes in the metabolism of the intact animal, induced by thyroid feeding or thyroidectomy, produce changes in the rate of oxidation of amino acids by kidney and liver.

Methods

Hyperthyroidism was produced in albino rats by thyroid feeding. Usually 2 grains of dried thyroid were fed daily for 5 to 7 days. Hypothyroidism was produced by thyroparathyroidectomy. The animals were used several months after the operation. Both sexes were employed. The animals weighed from 180 to 220 gm. when finally used. The control and hypothyroid rats were fasted from 18 to 20 hours before use, the hyperthyroid 10 to 12 hours. The metabolic rate of the sleeping, intact animal was determined by the method of Marquand (6).

---

* The data in this paper were taken from the thesis presented by J. R. Klein to the Graduate School of Arts and Sciences of Duke University in May, 1939, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Broken cell preparations of liver and kidney were prepared by grinding the tissues with sand and 0.06 M phosphate buffer (pH 7.8). The resulting mixtures were squeezed through coarse muslin.

EXPERIMENTAL

The rate of oxidation of *d*-amino acids by broken cell preparations of liver from control and hyperthyroid rats was determined. The preparations used were able to deaminate the amino acids but not able to oxidize the deaminated residues. The rates were determined by measuring the oxygen consumptions in the Warburg apparatus. The data obtained are presented in Table I.

Table I shows that the preparations made from the livers of hyperthyroid rats oxidize *d*-amino acids at a greater rate than those from the livers of control rats. This indicates that the increase in the rate of metabolism produced by thyroid feeding is accompanied by an increase in the concentration of the *d*-amino acid oxidase of rat liver.

The rate of oxidation of *d*-amino acids by broken cell preparations of liver from control and thyroidectomized rats was measured. A higher concentration of tissue (0.3 gm. plus 1 ml. of buffer) was used than in the experiments given in Table I (0.2 gm. plus 1 ml. of buffer). This was done in the hope of accentuating the difference between the rates with preparations from control and hypothyroid animals. The data obtained are presented in Table II. Since the rate of oxidation was found to be not directly proportional to the concentration of tissue, the data in Tables I and II are not directly comparable.

Table II shows that the rate of oxidation by liver of *d*-amino acids is decreased in hypothyroidism. This indicates that the decrease in the rate of metabolism produced by thyroidectomy leads to a decrease in the concentration of the *d*-amino acid oxidase in rat liver.

The rate of oxidation of *d*-amino acids by broken cell preparations of kidney from control, hyperthyroid, and hypothyroid rats was measured. No difference in the rate was found between the preparations of kidney from the control and the other groups. The concentration of tissue was 0.03 gm. plus 1 ml. of buffer. Broken cell preparations of kidney and liver oxidize, with a few exceptions, only *d*-amino acids. Slices of kidney and liver oxidize both *d*- and *l*-amino acids (7, 8). Therefore, in order to determine the effect of the change in the rate of oxygen consumption of the intact animal upon the rate of oxidation of *l*-amino acids, experiments with tissue slices were done. For comparison with the data obtained with broken cell preparations the rate of oxidation of *d*-amino acids by slices was also determined. Oxygen uptakes and urea production by liver slices in the presence of *d*- and *l*-amino acids were measured. While the data suggest that the liver slices from hyperthyroid rats oxidize both isomers of the

TABLE I

Rate of Oxidation of d-Amino Acids by Broken Cell Preparations of Liver from Control and Thyroid-Fed Rats

The concentration of liver was 0.2 gm. plus 1 ml. of buffer. Each vessel contained 1 ml. of this preparation. The concentration of amino acid was 0.018 M. The inset contained alkali. The final volume was 2 ml. The values for the oxidation of the amino acids are the differences between the oxygen uptakes with and without amino acids, expressed as c.mm. per hour.

Rat No.	Metabolic rate of intact animal	Amino acid			
		d(−)-Isoleucine	*d*(−)-Valine	*d*(−)-Alanine	*d*(+)-Phenylalanine
		Controls			
	ml. per hr. per kg.				
1	976	45	45	68	91
2	932	31	31	44	67
3	980	30	34	45	68
4	895	52	54	74	93
5	936	31	35	39	
Mean. . . .	974	38	40	54	61
		Hyperthyroid rats			
	1522	98	110	141	159
	1610	66	76	96	126
	1470	56	65	86	121
	1722	80	88	124	150
	1839	97	103	143	116
Mean.	1632	79	88	118	134

amino acids at a greater rate than those from control rats, the individual variations were too great to warrant definite conclusions. Experiments with slices of kidney cortex from the three groups of rats did not indicate any unequivocal differences in ability to oxidize *d*-, *l*-, or *dl*-amino acids.

TABLE II

Oxidation of d-Amino Acids by Broken Cell Preparations of Liver from Control and Thyroidectomized Rats

The concentration of liver was 0.3 gm. plus 1 ml. of buffer. The other details were the same as indicated in Table I.

Rat No.	Metabolic rate of intact animal	Amino acid			
		d(−)-Isoleucine	*d*(−)-Valine	*d*(−)-Alanine	*d*(+)-Phenylalanine
		Controls			
	ml. per hr. per kg.				
1	988	72	75	96	
2	948	98	100	128	
3	908	70	65	85	
4	893	75	78	85	
5	932	69	77	100	134
6	894	68	67	94	140
7	952	78	80	90	143
Mean......	930	76	77	97	139
		Hypothyroid rats			
1	750	52	57	69	
2	721	59	55	84	
3	709	50	53	62	
4	730	65	60	80	
5	765	60	63	78	120
6	788	59	58	75	123
7	824	59	58	75	118
Mean......	755	58	59	75	120

The addition of thyroxine or thyroglobulin *in vitro* did not change the rate of oxidation of amino acids by broken cell preparations, or by slices of kidney or liver.

The help and criticism given during the course of this work by Dr. W. A. Perlzweig is gratefully acknowledged.

CONCLUSIONS

The increase in the rate of metabolism of rats produced by thyroid feeding is accompanied by an increase in the concentration of *d*-amino acid oxidase of liver, but not of kidney. The decrease in the rate of metabolism following thyroidectomy is accompanied by a decrease in the concentration of *d*-amino acid oxidase in liver, but not in kidney.

BIBLIOGRAPHY

1. Dye, J. A., and Waggener, R. A., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **85**, 365 (1928).
2. Dye, J. A., and Waggener, R. A., *Am. J. Physiol.*, **85**, 1 (1928).
3. Gerard, R. W., and McIntyre, M., *Am. J. Physiol.*, **103**, 225 (1933).
4. McEachern, D., *Bull. Johns Hopkins Hosp.*, **56**, 145 (1935).
5. Cohen, R. A., and Gerard, R. W., *J. Cell. and Comp. Physiol.*, **10**, 223 (1937).
6. Marquand, C. B., Abstracts of doctor's dissertations, No. 22, 161, The Ohio State University Press (1937).
7. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **109**, 131 (1935).
8. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933); *Biochem. J.*, **29**, 1620 (1935).

STUDIES ON THE PRODUCTION OF TAUROCHOLIC ACID IN THE DOG

IV. CYSTEINE, HOMOCYSTEINE, AND THIOGLYCOLIC ACID*

By ROBERT W. VIRTUE AND MILDRED E. DOSTER-VIRTUE

(From the Department of Chemistry, University of Denver, Denver)

(Received for publication, February 27, 1939)

Results reported in the first paper of this series (1) indicated that either cystine or methionine may be the precursor of taurine in the dog. It is easily seen that decarboxylation and oxidation may change cystine to taurine. This has been done *in vitro* (2). The change from methionine to taurine seems more complex. Until quite recently it appeared that a possible explanation could consist of a replacement of cystine by methionine somewhere in the organism, thereby liberating a cystine molecule for transformation to taurine. The papers of Dawbarn (3) and of Beach and White (4), however, present fairly direct evidence that methionine may actually be changed to cystine in the body. If this change does occur, it appears that demethylation is probably the first step in the process. Such demethylation would produce homocysteine. This substance on oxidation would form homocystine. Evidence for the biological formation of homocystine from methionine has come from several sources (5). If both homocystine and homocysteine are changed to cystine, it is conceivable that they might both be able to form taurine. Our experiments, however, indicated that homocystine was not easily changed to taurine. This could be accounted for by assuming that homocysteine is the intermediate between methionine and cysteine, while homocystine followed some other path of oxidation. If this assumption is correct, homocysteine and probably cysteine should be able to form taurine. To obtain evidence concerning

---

* Grateful acknowledgement is made for the aid of a grant to one of the authors (R. W. V.) from the Committee on Scientific Research, American Medical Association.

this possibility it was decided that bile fistula experiments should be carried out with use of homocysteine and cysteine. Thioglycolic acid was used as a control sulfhydryl compound.

EXPERIMENTAL

It is accepted that cholic acid is the limiting factor in the formation of taurocholic acid (6). However, under proper conditions taurine may become the limiting factor. These conditions require that an excess of cholic acid over taurine be present in the liver. To obtain this excess, as in previous experiments, we depleted the livers of bile fistula dogs of their taurine by fasting, and by feeding 6.7 milliequivalents of cholic acid daily to each animal to cause formation of taurocholic acid from whatever taurine might be in the liver. The conjugated material was then presumably excreted in the bile. When the excretion of taurocholic acid had fallen off after 2 days of this régime, 6.7 milliequivalents of a sulfur-containing compound were given with the cholic acid on the 3rd day to see whether the dog could form taurine from the substance administered, and conjugate it with the cholic acid to be eliminated as extra taurocholic acid in the bile. The bile was collected at 24 hour intervals, just before the cholic acid was given. The urines were obtained by catheterization.

Homocystine was prepared from *dl*-methionine according to the modification by Brand *et al.* (7), of the method of Butz and du Vigneaud (8). This was reduced to homocysteine by heating with tin dust and hydrochloric acid. The tin was precipitated by passing in a stream of hydrogen sulfide, and the hydrogen sulfide was removed with a stream of carbon dioxide. The homocysteine solution so prepared showed a sulfhydryl content of from 96 to 102 per cent of the sulfur present, when titrated with iodine. Since these values represented a practically quantitative reduction of the homocystine, the amount of solution to be injected into the dogs was based on the sulfhydryl content as determined by iodometric titration. The solution was neutralized with sodium hydroxide just before injection. Cystine was prepared from human hair, and the *l*-cysteine used was prepared from the *l*-cystine by the method just described for the reduction of homocystine to homocysteine. The volume of cystine solution that was used was determined iodometrically, assuming a quantitative reduction of cystine to cysteine by the tin and hydrochloric acid. The thioglycolic acid was purchased from the Eastman Kodak Company. The commercial product was redistilled and analyzed for total sulfur by an alkaline fusion followed by a gravimetric determination as barium sulfate. The sulfhydryl content was determined iodometrically. The disulfide content was determined by reduction with zinc and hydrochloric acid followed by iodometric titration and subtraction of the sulfhydryl value. The composition of the material used was as follows:

$C_2H_4O_2S$.	Calculated.	S 34.75,	—SH 35.83,	—SS— 0.00
	Found.	" 34.98,	" 31.17,	" 1.43

The sulfhydryl content was 87 per cent of the theoretical value. Some difficulty was encountered on administration of this substance. The material was fed in a gelatin capsule after neutralization, but was promptly vomited. The next three times the thioglycolic acid was neutralized and injected intravenously, but in each case the effect was to cause vomiting of the cholic acid which had been fed. This occurred even when the injection of thioglycolic acid was delayed as long as 2 hours after feeding cholic acid, in which case the vomitus appeared to have come from the intestine. The successful experiments, for which protocols are given in Table I, were accomplished through use of subcutaneous injections of a neutralized solution of thioglycolic acid.

The analytical methods used have been described previously (1).

Results

Homocysteine—The results shown in Table I obtained from the urine of Dogs 39, 50, and 52 indicate that sulfur of homocysteine is rather readily oxidized to sulfate. Not a great deal of the sulfur of homocysteine was recovered, however, especially in the case of Dog 50. There will also be seen an increase in organic sulfur values on experimental days, but these increases were smaller (except with Dog 50) than those found in the sulfate fraction of the urine. No significant change in the excretion of disulfide sulfur was observed. The urinary sulfur partitions are much like those found after administration of homocystine to dogs (1). The taurocholic acid output was augmented after the administra-

TABLE I

Urinary Sulfur Partitions and Taurocholic Acid Production Following Administration of Cysteine, Homocysteine, and Thioglycolic Acid to Dogs

Each animal was fed 6.7 milliequivalents = 2.8 gm. of cholic acid daily.

Dog No.	Day	Weight	Total N	Total S	Sulfate S	Organic S	—SS— S	Tauro-cholic acid
		kg.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
39	1	12.0	3.45	142	41	101	5	1664
	2		3.36	177	82	95	4	320
	3		2.47	238	143	95	4	828*
	4	10.4	3.31	182	86	96	5	340
46	1	14.0	4.43	242	146	96	5	1246
	2		4.77	244	143	101	4	605
	3		4.04	306	196	110	5	1283*
	4	13.2	3.32	205	97	102	9	490
48	1	13.2	4.26	260	163	97	5	1485
	2		4.00	270	174	96	5	566
	3		3.84	341	235	106	7	1288†
	4	11.4	1.48	168	82	86	2	702
39	1	12.1	3.77	205	92	113	1	2157
	2		3.41	202	107	95	3	641
	3		2.87	271	157	114	4	1350‡
	4	11.1	3.33	232	111	121	6	503
50	1	15.0	5.97	396	229	167	6	1372
	2		6.22	393	279	114	4	805
	3		5.12	413	284	129	10	1397‡
	4	13.0	4.64	337	225	112	7	911
52	1	10.6	3.11	150	65	85	1	624
	2		3.97	170	84	86	0	346
	3		3.93	267	154	113	2	975‡
	4	9.8	3.93	188	104	84	7	631
54	1	14.5	10.24	462	321	141	11	3376
	2		5.88	314	194	120	7	2953
	3		6.52	501	342	159	12	1375§
	4	13.2	5.28	340	170	170	5	1245
55	1	15.9	5.85	319	204	115	7	2293
	2		5.40	280	171	109	4	1976
	3		5.75	548	398	150	18	1984§
	4	14.1	5.23	281	171	110	5	1610

* 6.7 milliequivalents of cysteine (0.8 gm.) were given orally after neutralization with sodium hydroxide.

† 6.7 milliequivalents of cysteine (0.8 gm.) were given intravenously after neutralization with sodium hydroxide.

‡ 6.7 milliequivalents of homocysteine (0.9 gm.) were given intravenously after neutralization with sodium hydroxide.

§ 6.7 milliequivalents of thioglycolic acid (0.65 gm.) were given subcutaneously after neutralization with sodium hydroxide.

tion of homocysteine to these animals. This finding is in distinct contrast to the effect produced by the administration of homocystine (1).

Cysteine—The administration of cysteine to bile fistula dogs was followed by results which are quite similar to those obtained after cystine (1). The urinary sulfur partition for Dogs 39, 46, and 48 in Table I show that most of the extra sulfur excreted on the experimental days appeared in the sulfate, or oxidized fraction. The excretion of taurocholic acid was augmented on administration of cysteine. No appreciable difference was noted in the excretion of disulfide sulfur.

Thioglycolic Acid—A greater percentage of the sulfur administered was recovered in the urine after giving thioglycolic acid than after administration of either cysteine or homocysteine. (See Hill and Lewis (9) for a discussion of recovery values greater than 100 per cent.) By far the larger part of this increase was in the sulfate fraction of the urine. Dogs 54 and 55 eliminated about 80 per cent of the extra sulfur as sulfate. Definite rises in excretion of disulfide sulfur appeared, but these account for only a small proportion of the sulfur administered, and are quite likely due to oxidation of the sulfhydryl form of some thioglycolic acid to the disulfide form. Thioglycolic acid, in contrast to cysteine and homocysteine, failed to cause an increased output of taurocholic acid in the bile. When administered subcutaneously after having been neutralized, the doses of thioglycolic acid we used caused a temporary local irritation but gave rise to no apparent toxicity.

DISCUSSION

Several reports that cystine and cysteine may have different paths of metabolism in the mammalian organism have appeared in the past few years. Brand, Cahill, and Harris (10) observed that a cystinuric patient was readily able to oxidize cystine to sulfate, but that the same patient excreted most of the cysteine fed as extra urinary cystine. Lewis, Brown, and White (11) made observations of a similar nature on a subject with cystinuria, although they found a smaller proportion of ingested cysteine excreted as urinary cystine than did Brand *et al.* More recently, Kassell, Cahill, and Brand (12) studied the metabolism of lactalbumin and of reduced lactalbumin in a case of cystinuria. A larger amount of extra cystine was excreted after feeding reduced lactalbumin which contained 2.5 per cent cysteine than after feeding lactalbumin which contained no cysteine. They conclude that, "Cystine and cysteine, as part of the same protein, behave as two different amino acids with separate catabolic pathways. There are probably separate mechanisms for the oxidation of S—S and —SH compounds."

Evidence supporting this last sentence was found in earlier work by Brand, Cahill, and Block (7) in which the metabolism of homocystine and homocysteine was investigated in a case of cystinuria. The sulfur of homocystine, like that of cystine, was almost completely oxidized to sulfate. Homocysteine, however, like cysteine, was excreted largely as extra urinary cystine. Our results support the contention of Brand that sulfhydryl and disulfide compounds have different paths of catabolism so far as homocystine and homocysteine are concerned. We find that homocysteine was apparently changed to taurine, while homocystine was not. If methionine is changed to taurine by demethylation as the first step, it would appear that oxidation of the homocysteine to homocystine must be prevented. Our observations do not support the idea that cystine and cysteine have different metabolic paths, for both substances gave rise to extra taurine. Since taurine formation is only one phase of sulfur metabolism, these results do not necessarily prove that cystine and cysteine may not be metabolized somewhat differently, nor are they necessarily in disagreement with the hypothesis that these two amino acids may be metabolized by different pathways in a cystinuric individual.

One outstanding question that arises here remains unanswered. If homocystine is changed to cystine in the course of metabolism, and if cystine gives rise to taurine, why does homocystine not give rise to taurine? It does seem likely that the series methionine-homocysteine-cysteine-taurine may be one path of catabolism of methionine.

Evidently some factor other than sulfhydryl stimulation is required for formation of taurine, for thioglycolic acid failed to increase the taurocholic acid output. Smith (13) in an early paper reported that about one-sixth of the sulfur of the ammonium salt of thioglycolic acid was oxidized by the dog. He fed 9 gm. of thioglycolic acid over a period of 3 days, and found the substance quite toxic. Hill and Lewis (9) observed that the sodium salt of thioglycolic acid, given orally or subcutaneously to rabbits, was toxic, but that slightly more than half of the extra sulfur recovered in the urine had been oxidized. Our dose per kilo was somewhat smaller than that used by either group of workers mentioned, and we found that the sulfur was as readily oxidized to sulfate as that of cystine.

SUMMARY

1. Cholic acid was fed to fasting bile fistula dogs for 2 days to enhance their output of taurocholic acid, and thereby deplete their livers of taurine. When homocysteine was injected intravenously on the 3rd day, at the same time the cholic acid was fed, the output of taurocholic acid increased.

2. When cysteine was fed or injected intravenously at the same time the cholic acid was fed, the excretion of taurocholic acid increased.

3. Thioglycolic acid injected subcutaneously at the time of feeding cholic acid failed to increase the excretion of taurocholic acid.

4. Under the conditions of our experiments the dog could evidently change homocysteine or cysteine to taurine, but could not form taurine from thioglycolic acid.

5. The greater part of the extra urinary sulfur from thioglycolic acid administered subcutaneously, from homocysteine given intravenously, or from cysteine given orally or intravenously was oxidized to sulfate by the dog.

The authors wish to thank Dr. R. W. Whitehead of the University of Colorado Medical School for his cooperation in making available facilities for carrying out these experiments.

BIBLIOGRAPHY

1. Virtue, R. W., and Doster-Virtue, M. E., *J. Biol. Chem.*, **119**, 697 (1937).
2. Friedemann, E., *Beitr. chem. Physiol. u. Path.*, **3**, 1 (1903). White, A., and Fishman, J. B., *J. Biol. Chem.*, **116**, 457 (1936).
3. Dawbarn, M. C., *Australian J. Exp. Biol. and Med. Sc.*, **16**, 159 (1938).

4. Beach, E. F., and White, A., *J. Biol. Chem.*, **127**, 87 (1939).
5. Virtue, R. W., and Lewis, H. B., *J. Biol. Chem.*, **104**, 59 (1934). Vars, H. M., *Proc. Soc. Exp. Biol. and Med.*, **31**, 129 (1933). Chase, B. W., and Lewis, H. B., *J. Biol. Chem.*, **101**, 735 (1933). Pirie, N. W., *Biochem. J.*, **28**, 305 (1934).
6. MacLeod, J. J. R., Physiology and biochemistry in modern medicine, St. Louis, 6th edition, 740 (1930).
7. Brand, E., Cahill, G. F., and Block, R. J., *J. Biol. Chem.*, **110**, 399 (1935).
8. Butz, L. W., and du Vigneaud, V., *J. Biol. Chem.*, **99**, 135 (1932–33).
9. Hill, R. M., and Lewis, H. B., *J. Biol. Chem.*, **59**, 557 (1924).
10. Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935).
11. Lewis, H. B., Brown, B. H., and White, F. R., *J. Biol. Chem.*, **114**, 171 (1936).
12. Kassell, B., Cahill, G. F., and Brand, E., *J. Biol. Chem.*, **125**, 423 (1938).
13. Smith, W. J., *Z. physiol. Chem.*, **17**, 459 (1893).

STUDIES IN MINERAL METABOLISM WITH THE AID OF ARTIFICIAL RADIOACTIVE ISOTOPES

II. ABSORPTION, DISTRIBUTION, AND EXCRETION OF POTASSIUM*

By MICHAEL JOSEPH, WALDO E. COHN,
AND DAVID M. GREENBERG

(From the Division of Biochemistry, University of California Medical School, Berkeley)

(Received for publication, March 20, 1939)

The use of artificial radioactive postassium offers a new means of studying the fate of this important mineral element in the animal organism. By means of potassium "labeled" in this manner it is possible to distinguish an administered dose of potassium from that already present in the animal under investigation. The advantages of using radioactive isotopes in the study of metabolism has been discussed in a recent review by one of us (2).

The present communication is concerned with a study of the absorption from the gastrointestinal tract, the rate of excretion via the urine, and the distribution in certain viscera of the body of potassium administered as potassium chloride.

The period of time over which it was possible to follow the fate of the labeled potassium was limited to a few days by the relatively rapid decay of K^{42} (half life, 12.8 hours). After several days, the emitted radioactivity becomes too low for accurate measurement. With a rapidly metabolized element such as potassium, however, the main picture of its disposition can be obtained within a relatively short time.

Methods

Preparation of Radioactive Potassium—The radioactive potassium (K^{42}) was prepared from metallic potassium by bombardment with deuterons in the cyclotron of the Radiation Laboratory of the University of California. The nuclear reactions involved are given below.

---

* A preliminary communication of this work has been published (1).

$${}_1H^2 + {}_{19}K^{41} \longrightarrow {}_{19}K^{42} + {}_1H^1$$

$${}_{19}K^{42} \longrightarrow {}_{20}Ca^{42} + e^- + \gamma \text{ radiation}$$

The bombarded potassium was dissolved off the target with alcohol in an atmosphere of CO_2 in order to avoid ignition. It was converted into its chloride by adding hydrochloric acid and then evaporating to dryness. The dry salt was redissolved in water and the potassium was separated from any contaminating sodium by precipitating it as potassium acid tartrate. It was then converted to potassium chloride by adding hydrochloric acid and igniting. The final solution of potassium chloride was made up to a concentration of 0.5 per cent, which gave a radioactive strength such that the amount in 1 ml. was 1000 to 5000 times as radioactive as the minimal quantity detectable with the Lauritzen electroscope.

The experimental work was carried out on normal rats, male and female, weighing between 200 and 350 gm. After a preliminary run it was found desirable to avoid the passage of feces during the experimental period because, even with the use of a device to separate feces from urine, it was found impossible to prevent completely the contact of the feces with the urine. Feces which came in contact with the urine soaked up sufficient quantities to lead to considerable error in estimating the urinary excretion of the labeled potassium. To clear the intestines and eliminate passage of feces, food was withdrawn for 48 hours prior to an experiment. During this period, three 10 ml. doses of Locke's salt solution, to which there had been added 6 per cent glucose, were administered.

To carry out an experiment, 1 ml. of the radioactive potassium chloride solution was administered by stomach tube or by intraperitoneal injection. In the case of the heavier animals, 2 ml. of the solution were used. The animals on which excretion was followed were placed in metabolism cages. Each cage stood over a glass funnel to the bottom of which was attached a urine-feces separator in order to collect the excreta (3).

At the proper time intervals after administration of the labeled potassium chloride, the animals were anesthetized with chloroform, bled by heart puncture, and the tissues to be analyzed were dissected out. In order to obtain the contents of the stomach and the small intestines, these organs were tied off separately, slit open, and the contents washed out with a stream of water from a wash bottle.

The radioactive potassium was extracted for analysis by covering the dissected tissue with 10 to 20 ml. of a 20 per cent trichloroacetic acid solution and then grinding it up with clean sand which had been washed previously with dilute hydrochloric acid in a glass mortar. To complete the extraction, the macerated tissue was repeatedly washed with distilled water. The filtered extract was transferred to a shallow 10 ml. Coors ashing capsule and evaporated to dryness on a hot-plate. The radioactivity of the sample was then measured on the electroscope. Tests for the completeness of the extraction were carried out very often by ashing the tissue residue and testing it for any residual radioactivity.

The radioactivity was measured in arbitrary units and then expressed in terms of the per cent of a standard sample of the initial radioactive potassium solution whose activity was determined at the same periods as the experimental samples. The radioactivity of the urine was determined in the same manner as that given for the tissue extracts.

Four series of experiments were carried out in this study. In Series I, radioactive potassium was administered by stomach tube to six rats which were sacrificed in pairs, 10, 20, and 30 minutes after the administration. In Series II, after oral administration, the six rats were sacrificed at the time intervals of 1, 2, 4, 7, 9, and 22 hours. In Series III, four rats were given intraperitoneal injections of the labeled potassium and were then sacrificed after ½, 1, 2, and 22 hours.

The excretion of the labeled potassium in the urine was determined on the animals of Series IV. Labeled potassium chloride was administered orally to one group of two animals and intraperitoneally to another group. The urine of each group was collected at intervals up to 66 hours and each urine collection was analyzed separately.

Results

Absorption—The curves showing the percentage of labeled potassium present in the fluid contents of the stomach and small intestine at varying intervals of time after its administration are plotted in Fig. 1. 10 minutes after administration, the stomach contents contained 43 per cent of the potassium and the small intestine contained 9 per cent, 48 per cent having been removed.

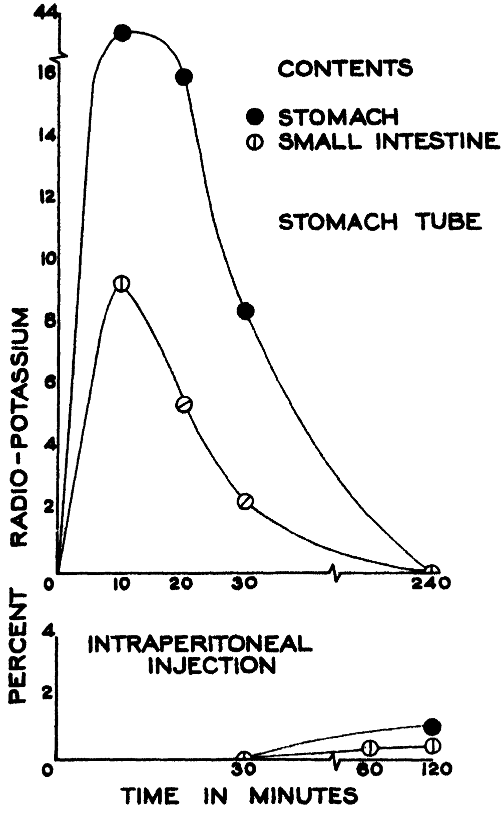


FIG. 1. Residual radioactive potassium in the gastrointestinal contents after oral administration of labeled potassium chloride and appearance of radioactive potassium in the contents following intraperitoneal injection of labeled potassium chloride.

About 90 per cent of the potassium was taken out within 30 minutes.

The rate of removal of the potassium was found to be subject to considerable individual variation. In one instance, 70 per cent of the administered potassium chloride was absorbed in the first 10 minutes.[1] This variation in the absorption rate was also reflected in the distribution of the radioactive potassium in the tissues.

Excretion of potassium into the stomach and small intestine is a minor factor in connection with these experiments. The curves given in the lower part of Fig. 1 show that not more than 1 per cent of intraperitoneally injected potassium ever appeared in the contents of the stomach and small intestine.

Distribution—During the height of absorption a large fraction of the administered potassium is present in the tissue of the small intestine. Fig. 2 shows that there was an average of 19.2 per cent at 10 minutes, and 23.7 per cent at 20 minutes after oral administration. These figures are subject to extreme individual variations of about ±50 per cent. The tissue of the stomach took up only about 10 to 20 per cent as much potassium as the small intestine. This observation is in harmony with the doctrine that the small intestine is the chief organ of absorption (4).

The absorbed potassium is taken up by the liver in a striking fashion. The curve of retention follows the absorption curve very closely, both in rise and fall. While the potassium is held in the liver for some time before it is released into the general circulation, the deposition there is only transitory and appears to be incidental to the location of the liver in the path of the portal venous system.

The preferential uptake of potassium by the tissue of the small intestine and liver occurs only when it is given orally. The lower part of Fig. 2 shows that, upon intraperitoneal injection of the labeled potassium chloride, the degree of accumulation of potassium by these organs is very much less. These observations appear to be readily explainable if the potassium is absorbed from the gastrointestinal tract into the portal venous system, which then carries it all through the liver.

Further evidence for this view-point is obtained when the labeled potassium uptake is plotted in terms of the per cent of the administered dose present per gm. of tissue rather than in terms of the per cent in the whole organ. Curves of the data have been plotted in this manner in Fig. 3. These curves show that initially the radioactive potassium content per gm. of liver is more than twice as great as the amount per ml. of blood in the general circulation. At the end of the absorption period the amount in the liver decreases, while the concentration in the blood increases to levels higher than that in the liver.

---

[1] The timing of the first 10 minute period was not very accurate because of the variations that necessarily occurred in chloroforming the animals, opening the abdomen, and dissecting out the stomach and small intestine.

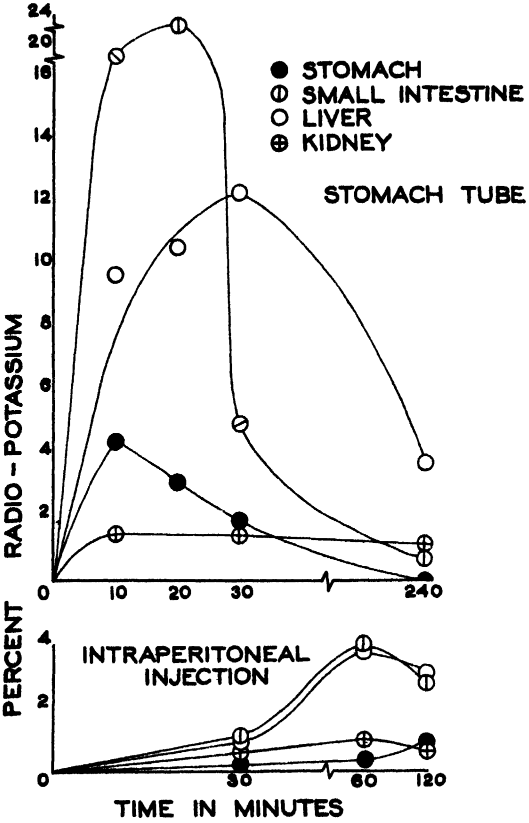


FIG. 2. Uptake of radioactive potassium by various organs following oral and intraperitoneal administration of labeled potassium chloride.

From Figs. 2 and 3 it appears that the kidney takes up a definite amount of potassium as soon as it appears in the circulation and maintains this constant level for a comparatively long time. Initially the amount is slightly higher per gm. of kidney than per ml. of blood, but the radioactive potassium content of the blood soon surpasses the concentration in the kidney.

The uptake of potassium by muscle is slow in comparison to the uptake of the other tissues considered here. The curve of uptake of radioactive potassium per gm. of muscle parallels the increase in the radioactive potassium of the blood. The quantity in the muscle during the period of uptake is maintained at about one-third the concentration in the blood.

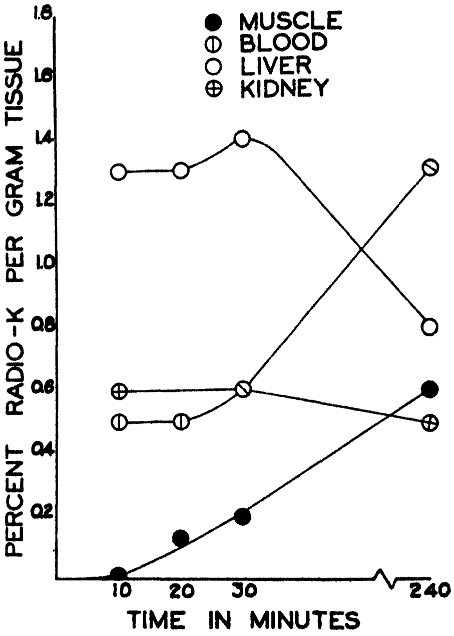


FIG. 3. The retention of radioactive potassium in various tissues and in blood per gm. of fresh weight.

After a number of hours the labeled potassium retention per gm. of fresh tissue (termed the "specific affinity" in a previous paper (5)) approaches the same value for such varied tissues as muscle, liver, kidney, stomach, small intestine, and heart.

Excretion—The urinary excretion of the labeled potassium is not very rapid and proceeds at the same rate regardless of whether it is given orally or by intraperitoneal injection. This is shown in Fig. 4. The curves for the urinary excretion of the labeled potassium are nearly identical regardless of the mode of administration. The excretion proceeds at a nearly constant rate of about 6 to 7 per cent per day for the 66 hours over which it was followed. The deduction to be drawn from this is that the absorbed potassium is rapidly taken up by the different tissues of the body, where it becomes mixed with and subsequently follows the same fate as the potassium already there. Under the conditions of the present experiments, the urinary excretion curves of the labeled potassium are a measure of the rate of the endogenous potassium metabolism of the rat.

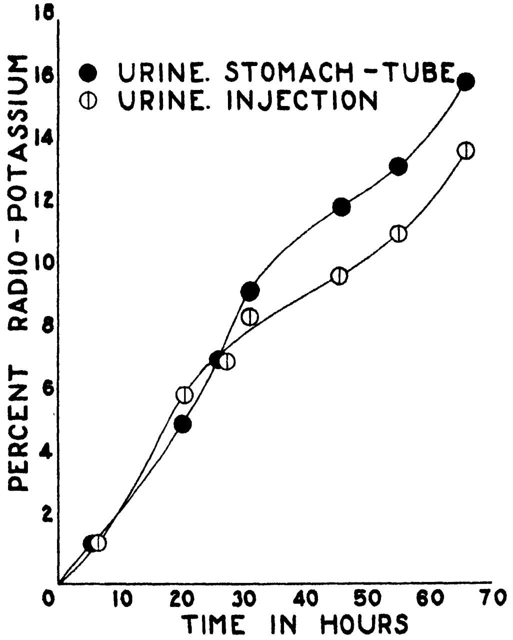


Fig. 4. Urinary excretion of radioactive potassium following oral or intraperitoneal administration of labeled potassium chloride.

DISCUSSION

The literature on the absorption of the mineral elements prior to the work with artificial radioactive isotopes has been summarized by Verzár (4). These studies have led to the conclusion that the absorption of ions was proportional to their rate of diffusion and that there was but little difference in the absorbability of the alkali elements. Using the increment in the radiation of the hand as a measure of absorption, Hamilton (6) has determined the rates of absorption of the radioactive isotopes of sodium, potassium, chlorine, bromine, and iodine in normal human subjects. He found that potassium ion was absorbed most slowly. All of the ions, except potassium, could be detected within 3 to 6 minutes following their ingestion, while it required 6 to 15 minutes for the absorption of potassium to be detected. At the end of 1 hour the curve for radioactive potassium had reached only 49 per cent of its final value, whereas the curves representing the absorption of sodium, chlorine, bromine, and iodine had risen to between 75 and 85 per cent of the maximum height. It would be inferred that the absorption of potassium from the human gastrointestinal tract is slower than it is from the rat. Allowance must be made, however, for the fact that the accumulation of potassium in the tissues of the stomach, intestine, and liver, such as is shown in Fig. 2, is not recorded by Hamilton's method of measurement, and would account for at least part of the slower absorption curve. Examination of Fig. 3 shows that the content of radioactive potassium in blood and skeletal muscle, which is more nearly comparable to Hamilton's measurements, is still increasing up to 6 hours after administration of the potassium.

The urinary excretion of labeled potassium in the present experiments occurred at a rather constant rate. There was no evidence of a diuresis or of a rapid initial excretion of potassium. Bock (7) observed a considerable diuresis and an increase in the rate of potassium excretion in the first few hours following intravenous injection of potassium chloride. The absence of an initial outpouring of potassium into the urine in the present experiments may be explained by the small quantities that were administered. Bock administered a considerable volume of isotonic potassium solution, whereas the quantity of labeled potassium administered in the present work was small.

SUMMARY

1. Radioactive potassium administered to fasted rats as potassium chloride is largely absorbed from the gastrointestinal contents within half an hour. There is a considerable degree of individual variation in the absorption rate, which suggests that there is no specific mechanism involved in the absorption of potassium ions.

2. The degree of accumulation of potassium in the tissue of the small intestine and stomach suggests that the absorption occurs mainly from the small intestine, with but little from the stomach.

3. Liver tissue shows an uptake of potassium which follows the absorption curve very closely both in rise and fall. The deposition in the liver is only transitory and appears to be incidental to its location in the path of the portal venous system.

4. The uptake of potassium by skeletal muscle is slow and increases continuously for at least 4 hours and then remains at a constant level for about 22 hours. The permeability of muscle for potassium is low. After 4 hours the content of labeled potassium per gm. of muscle was less than half of the concentration in the blood. The constant rate of increase of labeled potassium in muscle indicates the essential participation of potassium in muscle metabolism.

5. Excretion of labeled potassium through the kidney takes place at the moderate and nearly constant rate of about 6 to 7 per cent per day. The kidney accumulates a definite content of potassium as soon as it appears in the circulation and maintains it for a considerable length of time. Apparently the labeled potassium becomes mixed with and subsequently has the same fate as the potassium already present in the body. Under the conditions of the present experiments, the urinary excretion of the labeled potassium is a measure of the rate of the endogenous potassium metabolism of the body.

This investigation was aided by a grant from the Rockefeller Foundation and by Works Progress Administration laboratory assistance.

We gratefully acknowledge our indebtedness to Dr. Elma V. Tufts for assistance in part of this work, and to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for supplying us with the radioactive potassium used in these experiments. The work of the Radiation Laboratory is aided by a grant from the Research Corporation and by assistance of the Works Progress Administration.

BIBLIOGRAPHY

1. Greenberg, D. M., Joseph, M., Cohn, W. E., and Tufts, E. V., *Science*, **87**, 438 (1938).
2. Greenberg, D. M., in Luck, J. M., Annual review of biochemistry, Stanford University, in press.
3. Gross, L., and Connell, S. V. B., *J. Physiol.*, **57**, p. lx (1923).
4. Verzár, F., Absorption from the intestine, London (1936).
5. Cohn, W. E., and Greenberg, D. M., *J. Biol. Chem.*, **123**, 185 (1938).
6. Hamilton, J. G., *Am. J. Physiol.*, **124**, 667 (1938).
7. Bock, J., *Arch. exp. Path. u. Pharmakol.*, **57**, 183 (1907).

THE LOSS OF CARBON FROM EXCISED RHUBARB LEAVES DURING CULTURE*

By HUBERT BRADFORD VICKERY and GEORGE W. PUCHER

(*From the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, New Haven*)

(Received for publication, March 15, 1939)

When excised leaves are cultured in water or in nutrient solutions in darkness, prompt and extensive changes in composition take place. In general, unless an excess of sugar is supplied, there is a considerable loss of organic solids due to respiration, and it is usually assumed that the carbon dioxide eliminated is derived from the oxidation of the carbohydrates of the tissues (1).

The possibility that other components may also be involved has only in recent years received much attention. Yemm, for example, has shown (2) that the respiration of barley leaves, although apparently almost entirely a matter of carbohydrate oxidation during the first 24 hours of culture in darkness, subsequently draws upon other substances, and the behavior of the several nitrogenous fractions led him to infer that the products of protein hydrolysis were concerned. Our own studies of tobacco leaves (3, 4) have pointed to a similar conclusion.

Gregory and Sen (5) have recently studied the rate of respiration and its relationship to the chemical composition in a series of leaves of barley plants grown with complete control of the inorganic nutrients supplied. Each successive leaf was sampled as it attained full development and the data were subjected to a thorough statistical analysis. They found a high positive correlation between the respiration rate and the protein content in plants grown at different levels of deficiency of nitrogen and of potassium, with phosphorus maintained at a constant optimal level. Richards (6) extended the series to the study of potassium

---

* A portion of the expense of this investigation was borne by the Carnegie Institution of Washington.

and phosphorus deficiency with nitrogen constant and optimal, and has likewise observed a similar highly significant positive correlation. It was concluded that the respiration rates of young but fully developed leaves, under conditions of normal nutrition or of only moderate deficiency, are dependent almost entirely "on some aspect of their nitrogen metabolism, and are nearly independent of their carbohydrate level." Under conditions of extreme potassium or phosphorus deficiency the leaves are small and contain a lower proportion of protein and a higher proportion of amino nitrogen. With such abnormal leaves the content of reducing sugar plays an important part in determining the respiration rate. Richards pointed out that the simple view that protein nitrogen is merely a measure of the mass of protoplasm respiring, or of the enzyme systems concerned, is not adequate to account for the observations. The relationship between protein content and respiration is reciprocal, each being dependent on the other. Other components of the tissue that might be assumed to influence the rate of respiration varied between wide limits under the different nutritive conditions but were without effect upon this relationship.

Gregory and Sen have proposed as an interpretation of these findings a cyclic scheme of generalized reactions according to which protein synthesis and hydrolysis follow separate paths. The products of sugar decomposition are supposed to enter this cycle in part probably as organic acids which in turn give rise to amino acids and amides. Amino acids produced from the protein are oxidized more or less completely to carbon dioxide, ammonia, and water and provision is also made for carbon dioxide eliminated as a result of the decarboxylation of organic acids that arise from the sugar. The rate at which the cycle of protein synthesis and decomposition is traversed is held to be rapid. The ammonia set free by the oxidative deamination of amino acids from the protein combines with products of carbohydrate metabolism to form amides and these ultimately contribute to the synthesis of protein through amino acids, while the carbon residues that remain are respired away. The output of carbon dioxide is thus controlled by the rate at which the protein cycle is traversed and this determines the rate at which carbohydrates are oxidized. The carbon dioxide may or may not originate mainly from the amino acids, but in all cases there is a general regulatory process at work which governs the whole interrelated mechanism.

That proteins may be concerned in the respiration of plants is by no means a recent view. It was suggested as a possibility by Borodin (7) more than 60 years ago and has received occasional attention since that time (8, 9). In general, however, data in the literature suggest that respiration, during the period immediately after excision of the leaves, appears to draw almost exclusively upon the carbohydrates, the proteins being affected only after the lapse of considerable time.

EXPERIMENTAL

The study of the changes that take place in the composition of rhubarb leaves during culture under various conditions, certain phases of which have already been described (10), afforded us an opportunity to inquire into the effects upon the distribution of carbon among the different components of the tissue. The technique and analytical methods employed in these experiments have been indicated in previous papers (4). For the present study the total carbon of both dried blade and petiole tissues was determined by a slight modification of the wet combustion method of Friedemann and Kendall[1] (11). In addition the residual carbon was determined in samples of the same tissues from which soluble components had been removed by continuous extraction with hot 75 per cent alcohol. The soluble carbon could then be obtained by difference.

The results of these analyses for the samples of leaves collected in 1936 and 1937 and cultured under various conditions together with the total nitrogen values are shown in Tables I and II. The composition of the samples with respect to protein, glutamine amide, and other forms of nitrogen, organic acids, and carbohydrates had been determined and it was therefore possible to calculate the distribution of the carbon among the several components.

---

[1] The modifications consisted of the substitution of a more concentrated alkali solution (4 N) for the absorption of the carbon dioxide, and of the manometric method of Van Slyke for the determination of the carbonate. Difficulties from the distillation of acid fumes which might affect a titration method were thereby avoided. Samples of 0.100 gm. were oxidized for 45 minutes; control analyses of pure substances and of plant tissues of known carbon content gave satisfactory results, the reproducibility of the determinations being within 2 per cent of the quantity of carbon estimated.

The data for the changes, within certain arbitrarily selected intervals of time, of the carbon content of the components of the samples collected in 1936 are shown in Table III. In calculating the composition, the protein nitrogen was multiplied by the conventional factor 6.25 and the protein was assumed to contain 50 per cent of carbon. The soluble carbohydrate was assumed to have the same composition as glucose. The carbon of malic and citric acids was obtained from the direct determinations of these substances and the glutamine of protein origin was calculated on the assumption that rhubarb leaf proteins yield 8 per cent of their nitrogen in the form of glutamic acid[2] on acid hydrolysis but that this is liberated during intracellular enzymatic hydrolysis in the form of glutamine. The glutamine of other origin was calculated from the total glutamine amide nitrogen by deducting that from the protein. The "other alcohol-soluble" carbon was obtained from the total soluble carbon by subtracting the sum of the carbon of the carbohydrates, the glutamine, and the citric and malic acids.[3]

TABLE I

Carbon and Nitrogen Content of Rhubarb Leaves during Culture in Water in Darkness (1936 Series)

The figures are given in gm. per kilo of fresh weight of whole leaves.

Hrs.	0	24	72	165	261
Blade, total C	19.63	18.26	17.23	15.77	15.79
Petiole, total C	20.52	19.68	19.43	18.65	15.28
Blade, insoluble C	11.00	11.00	9.67	8.00	8.27
Petiole, insoluble C	9.66	9.66	9.39	10.12	9.08
Blade, total N	1.99	2.02	1.78	1.85	1.78
Petiole, total N	0.81	0.75	0.96	1.03	0.96

---

[2] This is an average value estimated from many closely agreeing analyses of various leaf proteins and was communicated to us by Professor Chibnall.

[3] This calculation involves the assumption that the whole of the citric and malic acids was extracted by the diluted alcohol. Tests have shown that this is not strictly the case but the error is not large and affects only the "other alcohol-soluble" carbon, making this a little low, and the "alcohol-insoluble non-protein" carbon, making this a little high.

Table II

Carbon and Nitrogen Content of Rhubarb Leaves during Culture under Various Conditions (1937 Series)

The figures are given in gm. per kilo of fresh weight of whole leaves.

Culture conditions*		DW			DG			LW		
Hrs.	0	25	93	165	25	93	165	25	93	165
Blade, total C	12.42	11.62	9.71	9.05	10.74	10.70	9.76	10.35	9.43	8.74
Petiole, total C	15.57	14.50	15.00	14.42	15.98	17.88	18.41	15.52	17.51	16.66
Blade, insoluble C	7.66	7.22	5.50	5.30	6.23	5.93	5.10	6.21	5.26	4.72
Petiole, insoluble C	7.75	7.48	7.54	8.04	7.24	8.30	8.90	7.79	8.90	8.82
Blade, total N	1.45	1.45	1.23	1.20	1.19	1.22	1.19	1.22	1.04	0.91
Petiole, total N	0.79	0.86	0.99	0.91	0.84	0.92	0.86	0.83	1.16	1.25

* The letter D indicates culture in darkness, L in light. The letters W and G indicate respectively culture in distilled water or in 0.3 M (5.4 per cent) glucose solution. The 0 hour sample furnishes a starting point for all three conditions of culture.

TABLE III

Changes in Carbon of Certain Components of Rhubarb Leaf Tissue during Successive Periods of Culture in Water in Darkness (1936 Series)*

The figures are given in gm. per kilo of original fresh weight of whole leaves.

	Blade				Petiole			
Culture period, *hrs.*	0-24	24-72	72-165	165-261	0-24	24-72	72-165	165-261
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Protein C	0.000	−1.15	−2.22	+0.12	−0.07	+0.06	+0.09	−0.23
Soluble carbohydrate C	−0.504	+0.044	−0.172	+0.176	−0.48	−0.59	−0.71	−0.17
Malic acid C	−0.171	+0.038	−0.074	−0.024	−0.17	−0.24	−0.67	−1.25
Citric " "	−0.008	−0.091	−0.044	−0.030	−0.006	+0.017	−0.022	−0.086
Glutamine " of protein origin	0.000	+0.127	+0.242	−0.014				
" " " other "	+0.018	+0.031	+0.431	−0.009	+0.005	+0.072	+0.137	−0.034
"Other alcohol-soluble" C	−0.70	+0.15	−0.17	−0.35	−0.19	+0.77	−0.25	−0.79
Alcohol-insoluble non-protein C	0.00	−0.18	+0.55	+0.15	+0.07	−0.33	+0.64	−0.81
Total C	−1.37	−1.03	−1.46	+0.02	−0.84	−0.25	−0.78	−3.37
	Whole leaf							
Total C	−2.21	−1.28	−2.24	−3.35				

* The time periods were selected to show the changes in the first 24 hours, the subsequent changes up to 72 hours of total culture time when yellowing of the blades first became apparent, the changes from 72 to 165 hours when the decomposition of the blade protein ceased and the leaves were beginning to become brown, and the later changes when the blade tissue was mostly brown and had ceased to respire and decomposition of the protein of the petiole finally became noticeable.

The alcohol-soluble fraction contains the relatively small amount of unknown organic acids, the soluble oxalic acid, the amino acids other than glutamine, and such components as the chlorophyll and yellow pigments, purines, betaines, bases, as well as other unknown soluble material. The "alcohol-insoluble non-protein" fraction was obtained by deducting the protein carbon from the insoluble carbon. It contains the cellulose, hemicellulose, pectins, and other complex substances of carbohydrate affiliations together with an appreciable quantity of material of unknown chemical nature.[4]

In the first 24 hours of culture, the leaves studied in 1936 lost 1.37 gm. of carbon from the blade and 0.84 gm. from the petiole or 2.21 gm. in all (Table III, Columns 1 and 5). The carbohydrate loss can account for only 0.98 gm. of carbon and it is clear that substances other than simple sugars were involved in the respiration even from the beginning. There was a large loss of "other alcohol-soluble" components from the blade, and this cannot be entirely accounted for as transport to the petiole since the "other alcohol-soluble" components of the petiole also diminished appreciably. The glutamine metabolism in this period made very small demands on the soluble carbon, and it is evident that the carbon lost from the leaves was due not only to oxidation of carbohydrates, but of other unknown alcohol-soluble components and probably of a little malic acid as well. The sum of the losses of these accounts quite accurately for the total carbon loss.

In the period from 24 to 72 hours, the loss of carbon from the blade was 1.03 gm., that from the petiole 0.25 (Columns 2 and 6). There was a loss of 1.15 gm. of protein carbon from the blade but this was only partly compensated by the gains of 0.127 gm. of glutamine carbon and of 0.15 gm. of "other alcohol-soluble" carbon. The "other alcohol-soluble" carbon of the petiole, however, increased by 0.77 gm., and the data for the nitrogen show that about 0.2 gm. of nitrogen was transported from the blade to the petiole. Accordingly transport of an appreciable quantity of carbon from the blade to the petiole probably also occurred and the changes can be considered only in terms of the whole leaf. The protein changes, however, took place almost exclusively in the blade. The loss of 1.28 gm. of carbon from the whole leaves probably includes the net loss of 0.54 gm. of carbohydrate carbon; the balance may have been made up by the 0.51 gm. of alcohol-insoluble carbon and the small malic acid loss. On the other hand, if some of the increased quantity of "other alcohol-soluble" carbon in the petiole were derived from the decomposition of the alcohol-insoluble non-protein fraction, *i.e.* the pectins, etc., it is quite possible that a part of the carbon of the protein may have been respired. It is impossible to decide between these views with the evidence at hand.

---

[4] Many of the data from which the carbon values were calculated have been given in two previous papers (10). The complete analytical data, together with the calculations of the carbon distribution from which the differences shown in Tables III and IV were obtained, are to be given in a forthcoming bulletin from this Station (12).

In the period from 75 to 165 hours, the loss of carbon from the blades was 1.46 gm. and from the petioles 0.78 gm. (Columns 3 and 7). The loss of carbohydrate carbon was 0.88 gm., mostly from the petiole, and accordingly, if this were entirely due to respiration, it remains to find the origin of the 1.36 gm. of carbon that also disappeared. A clue to what may have happened is provided by the glutamine metabolism. The increase in glutamine in this period was rapid and amounted to 0.43 gm. of carbon in the blade and 0.14 gm. in the petiole other than that which may have been derived directly from the protein. If this newly synthesized glutamine arose from the reaction of ammonia with products of the metabolism of the organic acids and carbohydrates,[5] the whole of the carbon of the malic and citric acids and of the soluble carbohydrate of the blades would be needed. Thus the loss of 1.46 gm. of carbon from the blade due to the combined effects of transport to the petiole and of respiration can be accounted for only if it is assumed that much of the 2.2 gm. of protein carbon that disappeared as such was involved. It is thus difficult to escape the conclusion that an appreciable part of this protein carbon contributed to the respiration in the blade. With respect to the petiole, either the malic acid or the carbohydrate alone would have been sufficient to account for nearly all of the loss of 0.78 gm. of carbon. It is probable that both were concerned.

---

[5] Hypotheses to account for this have been described by Chibnall (13) and are discussed in the light of the present data in another publication (12).

In the last period, from 165 to 261 hours, loss of carbon from the blade ceased. The blade tissue became brown at this time and this is usually taken as evidence of death of the cells; the cessation of any detectable loss of carbon from the blades either by transport, or by respiration confirms this view. In the petiole, on the other hand, the carbon loss was no less than 3.3 gm. The detailed data suggest that several components contributed to this loss and it seems evident that nearly half of it came from the oxidation of malic acid.

The chemical changes involved in the respiration of these leaves are obviously complex. In the early stages of the culture, it is clear that the carbohydrates carried a considerable share of the load but by no means all of it. Direct participation by the protein was not definitely evident until late in the period studied in this particular experiment, although it is possible that deamination products may have been drawn upon fairly early. In general, however, the results with this series of leaves do not emphasize this conclusion and resemble more closely those obtained by Deleano in his work with vine leaves.

The results of the carbon determinations on the material collected in 1937 were strikingly different. These leaves contained a much smaller proportion of soluble carbohydrates initially and the respiration drew upon a wide variety of the blade components even in the first 25 hours. The data of three experiments carried out respectively in water in darkness (DW), in 0.3 M glucose in darkness (DG), and in water in light (LW), are shown in Table IV. In the DW experiment, during the initial period of 25 hours, the carbohydrates could have supplied only about one-fifth of the carbon loss from the blade of 0.80 gm. (Column 1) and similar small proportions may have been derived from the "other alcohol-soluble" and from the alcohol-insoluble components. In spite of a digestion of protein to the extent of 0.25 gm. of carbon, the "other alcohol-soluble" fraction decreased. There was a small net loss of glutamine and appreciable losses of organic acids took place. Thus it is quite possible that some of the carbon compounds derived from the protein may have been completely oxidized even at this early stage of the culture. In the petiole

TABLE IV

Changes in Carbon Content of Components of Rhubarb Leaves in Successive Periods of Culture under Various Conditions

The figures are gm. of carbon in each component per kilo of original fresh weight of whole leaves.

Culture conditions*........................	DW			DG			LW		
Hrs..	0-25	25-93	93-165	0-25	25-93	93-165	0-25	25-93	93-165
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
					Blade				
Protein C........................	−0.250	−1.350	−0.780	−0.800	−0.570	−0.930	−0.770	−0.950	−0.710
Soluble carbohydrate C..........	−0.140	−0.048	+0.060	−0.012	−0.112	0.000	−0.100	−0.028	+0.004
Malic acid C.....................	−0.115	−0.075	+0.029	−0.063	−0.079	−0.078	−0.079	−0.011	−0.052
Citric " ".....................	−0.033	−0.049	−0.012	−0.048	−0.002	−0.050	−0.058	−0.018	−0.028
Oxalic " ".....................	+0.090	−0.098	+0.040	−0.013	+0.042	−0.034	−0.021	−0.037	+0.012
Glutamine " of protein origin....	+0.027	+0.148	+0.085	+0.087	+0.073	+0.100	+0.084	+0.104	+0.078
" " " other "	−0.053	+0.004	+0.262	−0.078	+0.074	+0.114	−0.068	+0.044	−0.012
Other alcohol-soluble C..........	−0.130	−0.070	−0.920	−0.120	+0.260	−0.160	−0.380	−0.020	−0.150
Alcohol-insoluble non-protein C..	−0.190	−0.370	+0.580	−0.630	+0.270	+0.100	−0.680	0.000	+0.170
Total C........................	−0.794	−1.908	−0.656	−1.677	−0.044	−0.938	−2.072	−0.916	−0.688
					Petiole				
Protein C........................	−0.095	+0.020	+0.015	−0.150	+0.075	+0.095	−0.025	+0.170	+0.140
Soluble carbohydrate C..........	−0.420	−0.232	−0.169	+0.440	−0.140	−0.020	−0.428	−0.176	−0.128
Malic acid C.....................	0.000	−0.150	−0.430	+0.300	+0.180	−0.440	+0.570	−1.010	−0.450
Citric " ".....................	−0.003	+0.002	+0.003	+0.025	+0.026	+0.022	+0.005	+0.027	−0.007
Oxalic " ".....................	+0.011	+0.003	+0.042	−0.034	+0.066	−0.005	+0.016	+0.029	−0.034
Glutamine ".....................	−0.041	+0.046	−0.025	+0.022	−0.007	+0.126	−0.083	+0.108	+0.115

Other alcohol-soluble C	−0.360	+0.780	−0.500	+0.160	+0.710	+0.260	−0.180	+1.910	−0.260
Alcohol-insoluble non-protein C	−0.170	+0.040	+0.480	−0.360	+0.990	+0.500	+0.070	+0.940	−0.220
Total C	−1.078	+0.509	−0.584	+0.403	+1.900	+0.538	−0.055	+1.998	−0.844
Whole leaf									
Total C	−1.87	−1.40	−1.24	−1.27	+1.86	−0.40	−2.13	+1.08	−1.53
Glucose C absorbed				+1.91	+2.65	+0.66			

* For explanation of the letters see the foot-note to Table II.

nearly half of the carbon loss of 1.08 gm. was clearly derived from the carbohydrates, the balance being made up from the two unknown fractions. The malic acid, however, was not drawn upon at all.

In the second period (Column 2), the blades lost 1.91 and the petioles gained 0.51 gm. of carbon; transport of a considerable amount of organic substance must therefore have occurred. The nitrogen data indicate that after 93 hours approximately 0.2 gm. of nitrogen had been transported along with this carbon. If it be assumed that the carbohydrate that disappeared from the leaves was respired, the net loss of total carbon shows that somewhat more than 1 gm. of carbon in forms other than carbohydrate was consumed in this way. The protein of the blade lost 1.35 gm. of carbon of which 0.15 probably remained as glutamine. The "other alcohol-soluble" carbon in the blade diminished slightly instead of increasing proportionately to the protein loss, and, accordingly, about 1.1 gm. of protein carbon must have been either transported to the petiole or respired. The "other alcohol-soluble" carbon of the petiole did increase by 0.78 gm. and this may furnish a measure of the amount of protein carbon transported, but the deficit of 0.32 gm. of protein carbon could scarcely have had any fate other than respiration. This estimate is minimal unless it is also assumed that carbon of protein origin was transported to the petiole, there to be converted into alcohol-insoluble substances.

In the final period (Column 3) the loss of 1.24 gm. of carbon was about equally shared by the blades and the petioles. There was little evidence of transport from the nitrogen data and none from the carbon data. In this period, glutamine was synthesized in considerable amounts in the blade but the losses of organic acids and carbohydrates were far too small to account for the change and it seems likely that substances included in the "other alcohol-soluble" fraction were drawn upon. If, in addition, the increase in "alcohol-insoluble" carbon were derived from this fraction, the greater part of the loss of 0.78 gm. of protein carbon must have been due to respiration. On the other hand, if the protein contributed to the alcohol-insoluble material, a smaller part of it was respired. In either case, however, some of the protein carbon must have contributed to the respiration. In the petiole it is clear that the carbon other than carbohydrate carbon lost may have been derived from either or both the malic acid and the "other alcohol-soluble" substances.

Consideration of the data for the leaves cultured in glucose solution is complicated by the advent of sugar. The quantities of carbon that entered the leaves in this form, as determined by analysis of the culture solution, are shown at the bottom of Table IV (Columns 4 to 6). In the first 25 hours, although 1.9 gm. of new carbon entered, the leaves lost 1.27 gm.; hence the total amount of carbon respired must have been 3.2 gm. There is evidence that nitrogen was transported from the blade to the petiole in this interval and the failure of the soluble carbohydrate of the blade to diminish suggests that some of the newly acquired glucose may have reached the blade. Accordingly it is difficult to disentangle the changes due to transport from those due to respiration. However, it is clear that hydrolysis of the blade protein was greatly stimulated as compared with that of the protein in the DW culture, but there was no corresponding increase in the carbon of soluble components in the blade; these in fact diminished. Thus the 1.68 gm. of carbon lost from the blade were mostly drawn from the protein and the alcohol-insoluble fraction. The increases in carbohydrate and malic acid in the petiole are obvious results of the glucose metabolism in this organ, but of the 1.91 gm. of new glucose carbon only 0.74 gm. can be thus accounted for, and the fate of 1.17 gm. remains to be decided. Even if this were all respired, 2 gm. of additional carbon from the original components of the leaves must also have been completely oxidized and it seems highly probable, therefore, that much of the loss of protein carbon was due to respiration. The possibility that the protein was drawn upon for respiration in spite of the presence of glucose derived from the culture solution is especially noteworthy.

In the period from 25 to 93 hours the leaves acquired 2.65 gm. of glucose carbon and increased in net carbon by 1.86 gm. The respiration loss was therefore only 0.8 gm. and it is difficult to allocate this small quantity. The soluble carbohydrate of both blade and petiole diminished but the increases in the other components show that the glucose either entered into or stimulated many complex reactions. The blade protein diminished and it is possible that some of the products of hydrolysis were respired, although this cannot be demonstrated from the data.

In the final period (Table IV, Column 6), although glucose absorption continued, there was a net loss of carbon and the total respiration loss must have been of the order of 1 gm. of carbon. A part of this was probably contributed by the malic acid of the petiole but most of the glucose must have been used in the petiole to form soluble and insoluble components. The blades lost 0.94 gm. of carbon mostly derived from the protein and there seems little doubt that a considerable part of this loss was due to respiration. There was no indication of transport of nitrogen from the blade, so that transport of any substantial amount of carbon is improbable.

The data for the leaves cultured in light (Table IV, Columns 7 to 9) include no measure of the carbon acquired by photosynthesis and interpretation in detail is therefore less satisfactory. In the first 25 hours, in spite of photosynthesis, the leaves lost 2.13 gm. of carbon, nearly all from the blade, and the actual respiration loss must have been appreciably larger. Transport of nitrogen and of carbon out of the blade was especially active. The petiole gained 0.57 gm. of malic acid carbon, probably derived from the metabolism of products of photosynthesis or from the carbohydrate. The combined losses of the carbon of unknown alcohol-soluble and insoluble components were 1.2 gm. and the loss of carbohydrate carbon was 0.53 gm. The latter quantity is offset by the gain in malic acid. Thus about 0.8 gm. of carbon lost from the leaves must have had some other source. The loss of protein carbon was 0.77 gm. and it seems clear that much of it must have entered into the respiration even at this early stage of culture in light.

In the period from 25 to 93 hours (Column 8) photosynthesis predominated over respiration and a considerable accumulation of various components occurred in the petiole. The loss of 0.92 gm. of carbon from the blade was chiefly due to the loss of 0.95 gm. of protein carbon. Doubtless much of this was transported to the petiole but the significant point is that although these leaves were supplied with products of photosynthesis, the blade protein was nevertheless rapidly hydrolyzed. There is no clue to the amount of carbon lost by respiration, but the cessation of the initial rapid loss of alcohol-soluble and insoluble products that took place in the 1st day shows that the general course of metabolism must have undergone a marked change. The synthesis of a moderate quantity of protein in the petiole is particularly striking.

In the last period (Column 9), respiration again outstripped photosynthesis and the leaves lost as much as 1.5 gm. of carbon. Transport of considerable nitrogen and probably also of carbon from the blade continued and regeneration of protein in the petiole was still detectable. Of the net loss of carbon, only 0.13 gm. could have been derived from carbohydrate and the losses can be accounted for only to the extent of 0.86 gm. by assuming that the malic acid and "other alcohol-soluble" components were respired. Even if 0.2 gm. of carbon from the alcohol-insoluble fraction in the petiole is included, it is still necessary to assume that about 0.5 gm. of protein carbon from the blade was also involved in the respiration.

DISCUSSION

The present analytical results indicate that the respiration of detached leaves in culture is an extremely complex phenomenon. The exact course of the respiration with respect to the nature of the tissue components that are oxidized may vary widely even from the beginning of the culture period according to the initial composition and the conditions. Although carbohydrates seem indeed to be the first point of attack, they supply only a fraction of the total loss of carbon. When, as in the 1936 samples, the initial supply of carbohydrates is reasonably high, it is difficult to demonstrate that the protein shares in the oxidation reactions until many hours have elapsed, although hydrolysis of the protein may and usually does begin quite early (4). On the other hand, if the leaves are initially somewhat deficient in carbohydrates, as was the case with the 1937 samples, it is possible to show that the hydrolytic products of the protein soon contribute to the loss of carbon dioxide. It is also especially striking that, even when glucose is supplied in culture in the dark or when products of photosynthesis are made available by exposing the leaves to light, hydrolysis of the blade protein likewise occurs and may even be stimulated and the products of hydrolysis are also drawn upon in the same way. It would appear that the residues that remain after the preliminary oxidative deamination of the amino acids are to only a limited extent available for subsequent synthetic reactions and are accordingly completely oxidized presumably by mechanisms analogous to those of ordinary fatty acid oxidation.

These findings correspond in general outline with the views of the chemical mechanism of leaf respiration suggested by Gregory and Sen. If it be assumed that, in the normal leaf, protein synthesis balances decomposition, only the share in the general scheme of reactions borne by the organic acids and especially the carbohydrates is obvious. In the detached leaf, however, protein synthesis is apparently much restricted or may even be suspended entirely. Thus the composition of the tissue soon becomes, as it were, distorted and reactions of kinds that are not usually regarded as characteristic of normal tissue may become evident.

The effects of respiration in the blade tissue were quite different from those in the petiole. The blades are provided with a relatively large proportion of protein and, regardless of any hypothesis of continuous decomposition and regeneration, this entered into reaction early in the culture period. In the petioles, on the other hand, the proportion of protein is much less and the quantity present seems to have been scarcely affected during culture in darkness for many hours while in light there was a definite increase. In general the carbon dioxide eliminated from the petiole tissue seems to have arisen from the soluble carbohydrates, the malic acid, and, in part, also from alcohol-soluble substances of unspecified chemical nature. Even the alcohol-insoluble complex carbohydrates may have contributed.

The increase in the protein content of the petioles of the leaves cultured in light, a phenomenon that has also been observed in the petioles of nasturtium leaves by Michael (14), may have significance in connection with the respiration if the views of Gregory and Sen may be applied to this tissue. It would appear that rhubarb petiole tissue, even after excision from the plant, retains a considerable capacity to bring about protein synthesis. In the petioles of the leaves cultured in darkness, this capacity was sufficient to balance the hydrolyzing activities quite closely. In light, on the other hand, the supply of products of photosynthesis, which led in the period between 25 and 93 hours (Table IV, Column 8) to an increase of about 2 gm. of carbon in spite of possible respiration losses, provided an opportunity for the synthesizing mechanisms to outstrip the hydrolysis and this continued even into the final period. Although respiration in the blades of these leaves probably did draw to some extent on carbon residues of protein origin, in the petiole the carbon lost was undoubtedly supplied by the consumption mainly of carbohydrates and organic acids.

Gregory and Sen have given their views only in the form of a highly generalized schema without specification as to the exact nature of the chemical reactions that may be involved. At the present time, when analytical data are expressed in terms of gm. of carbon or of nitrogen belonging frequently to substances of only vaguely recognized chemical character, a more precise form of expression is perhaps hardly justified. Nevertheless such attempts as those of Chibnall (13) to apply the Krebs and Johnson citric acid cycle (15) to the explanation of some of the details of plant cell respiration indicate the direction in which further progress is to be made. The present results with rhubarb leaves clearly indicate that the full explanation when obtained will be far from simple.

SUMMARY

The results of determinations of total carbon and of insoluble carbon in a series of leaves of the rhubarb plant are discussed in terms of the losses of carbon due to respiration that take place when the leaves are subjected to culture under various conditions. It is shown that respiration draws upon components other than carbohydrates even when leaves of reasonably high initial carbohydrate content are taken. With leaves of low initial carbohydrate content it is possible to demonstrate that an appreciable part of the carbon lost by respiration originated from the proteins of the blade tissue. These were hydrolyzed promptly in all cases and it is suggested that the residues that remain after oxidative deamination of the amino acids may be more or less completely oxidized subsequently.

Culture of the leaves in glucose solution or in water in light had little effect upon the rate of hydrolysis of the protein save possibly

to stimulate the initial phase, and, in spite of the additional supplies of carbohydrates thus furnished to the leaves, evidence that a part of the protein carbon was respired could still be obtained. Thus even when carbohydrate is supplied to the leaves, respiration may still draw upon a wide assortment of components of the tissues.

Speculative and highly generalized relationships between carbohydrates, organic acids, and proteins on the one hand and respiration on the other hand have been recently suggested by Gregory and Sen. The present results are found to be in general agreement with the views they have expressed, although no information with respect to detailed chemical mechanisms has been secured.

BIBLIOGRAPHY

1. Spoehr, H. A., and McGee, J. M., *Carnegie Inst. Washington, Pub. No. 325* (1923).
2. Yemm, E. W., *Proc. Roy. Soc. London, Series B*, **117**, 483 (1935); **123**, 243 (1937).
3. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Carnegie Inst. Washington, Pub. No. 445* (1933).
4. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agric. Exp. Stat., Bull. 399* (1937).
5. Gregory, F. G., and Sen, P. K., *Ann. Bot.*, **1**, 521 (1937).
6. Richards, F. J., *Ann. Bot.*, **2**, 491 (1938).
7. Borodin, I., *Bot. Ztg.*, **36**, 802 (1878).
8. Deleano, N. T., *Jahrb. wissensch. Bot.*, **51**, 541 (1912).
9. Meyer, A., *Flora*, **111-112**, 85 (1918).
10. Vickery, H. B., Pucher, G. W., Leavenworth, C. S., and Wakeman, A. J., *J. Biol. Chem.*, **125**, 527 (1938). Pucher, G. W., Wakeman, A. J., and Vickery, H. B., *J. Biol. Chem.*, **126**, 43 (1938).
11. Friedemann, T. E., and Kendall, A. I., *J. Biol. Chem.*, **82**, 45 (1929).
12. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agric. Exp. Stat., Bull. 424* (1939).
13. Chibnall, A. C., Protein metabolism in the plant, New Haven (1939).
14. Michael, G., *Z. Bot.*, **29**, 385 (1935).
15. Krebs, H. A., and Johnson, W. A., *Enzymologia*, **4**, 148 (1937).

THE METABOLISM OF AMIDES IN GREEN PLANTS*

III. THE MECHANISM OF AMIDE SYNTHESIS

By HUBERT BRADFORD VICKERY and GEORGE W. PUCHER

(*From the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, New Haven*)

(Received for publication, March 15, 1939)

The explanation of the formation of amides in green plants is one of the oldest problems of plant biochemistry. Since the discovery in 1806 of asparagine in asparagus shoots by Vauquelin and Robiquet (1) and the demonstration in 1848 by Piria (2) of the derivation of this substance in seedlings from the proteins of the seeds and of its chemical relationship to malic acid, asparagine and its later discovered homologue glutamine (3) have been repeatedly investigated. Schulze (4) in 1898 expressed the view that these substances arise in seedlings from the reaction of ammonia, derived from the amino acids of the seed protein, with nitrogen-free nutrients, and that they serve as easily transportable substances eminently suited to provide nitrogen for protein regeneration in the growing parts of the plant. Prianischnikow and Schulow (5) in 1910 first suggested that an important function of the amides was to provide a means for the disposal of ammonia which otherwise might accumulate in high concentration and prove toxic. This idea was later developed in considerable detail (6, 7) and was supported by remarkably able experimentation with seedlings grown in nutrient solutions that contained ammonia ((8) pp. 762–767).

Schulze's view that the carbon compound which combines with ammonia to form the amide has its origin from carbohydrates was given direct support by the pioneer work of Suzuki (9) with fully developed plants and the extensive labors of Prianischnikow with seedlings confirmed this assumption, but the details of the

---

* A portion of the expense of this investigation was borne by the Carnegie Institution of Washington.

chemical mechanism of the conversion remained obscure, although several investigators (5, 10) had made the suggestion that succinic, malic, or fumaric acid may be the intermediate compound concerned. A direct experimental attack on this aspect of the problem was made in 1933 by Mothes (11) who infiltrated solutions of the ammonium salts of these and of various other related organic acids into leaves and observed an increase in asparagine amide nitrogen. Schwab (12, 13) later showed, however, in carefully controlled parallel experiments, that ammonium sulfate was as effective as the salts of the organic acids in this respect and maintained that, although the ammonia was undoubtedly a determining factor in amide synthesis, there was no convincing evidence that the organic acid supplied actually furnished the carbon chain of the newly formed asparagine.

In a recent discussion of this problem (8) it was pointed out that present day views of intermediary metabolism indicate that the most probable immediate precursor of an amino acid is the corresponding α-keto acid (14). Thus the search for the precursors of the two amides narrows to a search for mechanisms whereby oxaloacetic acid and α-ketoglutaric acid may be produced in the tissues. These, in the presence of ammonia and of suitable enzyme systems, would be expected to yield respectively asparagine and glutamine.

Chibnall, in his exhaustive discussion of protein metabolism in plants (15), has considered amide metabolism from this point of view and has suggested the citric acid respiration cycle of Krebs and Johnson (16) as a chemical mechanism whereby both oxaloacetic acid and α-ketoglutaric acid may be rendered available for reaction with ammonia. According to this conception carbohydrates undergo transformation to 3-carbon products of which pyruvic acid is the most important for the present purpose. This is supposed to combine with oxaloacetic acid to form citric acid (17) which is then transformed through aconitic and isocitric acids to α-ketoglutaric acid (18). This in turn is oxidized by the Warburg-Keilin system to succinic acid which is subsequently transformed through malic and fumaric acids back to oxaloacetic acid (19). If such a system of enzymatic transformations is indeed at the basis of carbohydrate respiration, it is clear that a mechanism is provided whereby carbohydrate enters the cycle via pyruvic acid, oxygen enters at the transformation of α-ketoglutaric acid to succinic acid, and carbon dioxide is eliminated during several of the interconversions as the result of decarboxylation reactions. Each of the individual components of the system has been shown to stimulate the respiration of various animal tissues and many of the transformations have been demonstrated to be brought about by enzyme systems present in both plants and animals. Furthermore nearly every intermediate considered is more or less well known as a component of plant tissues, in particular in leaves.

This scheme provides an important clue to the solution of the problem of amide metabolism. If it or some analogous series of transformations actually does take place in plants, the mechanism for the formation of the most probable immediate precursors of the two known amides becomes clear. Furthermore these precursors arise from the carbohydrates through the organic acid metabolism, a concept that is in agreement with many experimental observations on the formation of amides. The most important point is, however, that the production of these substances is a function of the respiration and this idea throws a clear light on many findings that have hitherto proved incomprehensible.

On this view the emphasis upon amide formation as a mechanism to maintain ammonia at a low level—the "detoxication" hypothesis of Prianischnikow—is entirely removed. Although this hypothesis has been very useful in attempts to interpret the behavior of the amides in many species (8, 11, 13, 20, 21), it does not account for the behavior of ammonia and of glutamine in the rhubarb leaf (22, 23). Specimens of this plant examined in this laboratory contained when collected 15 per cent or more of their total nitrogen in the form of ammonium ions. The proportion was somewhat increased during culture of the excised leaves and there was a considerable increase in glutamine content, but no evidence was secured that the ultimate death of the cells had anything to do with the small increase in ammonia or that synthesis of glutamine was stimulated in any striking fashion by the high concentration of ammonium ions present.

Even the tobacco plant under certain conditions may contain a high proportion of ammonia without a corresponding increase in the concentration of amide nitrogen. Thus, plants grown in sand culture with a complete nutrient solution that supplied the nitrogen as nitrate contained, in the leaves, 2.25 per cent of the soluble nitrogen (exclusive of nitrate nitrogen) as ammonia and 5.06 per cent as amide nitrogen (asparagine and glutamine in about equal proportions). When grown with an otherwise similar culture solution in which 60 per cent of the nitrogen was supplied as ammonia and 40 per cent as nitrate, the plants were equally large and healthy but the ammonia nitrogen was 10.3 per cent of the water-soluble nitrogen although the amide nitrogen remained essentially unchanged at 5.5 per cent. When the proportion of the nitrogen supplied as ammonia was increased to 80 per cent, the plants were slightly smaller but the ammonia nitrogen in the leaves was increased to 26.6 per cent of the soluble nitrogen, while the amide nitrogen had increased to only 6.8 per cent. Thus normal healthy plants of tobacco may contain any proportion up to 26 per cent of the soluble nitrogen as ammonia when freshly collected without significant effect upon the level of the amide nitrogen. The plants grown with a high proportion of ammonia in the nutrient solution resembled rhubarb leaves to some extent in their nitrogenous composition but were by no means to be classified as "acid plants" (24). The reaction of the extract from the leaves was at pH 5.21 as compared with 5.47 for the nitrate controls.

Thus it seems clear that Prianischnikow's view that the synthesis of amides results from the effort of the cells to maintain a low level of ammonia nitrogen is not in accordance with the observations on rhubarb leaves or upon certain specially grown tobacco leaves analyzed immediately after collection. Furthermore it does not suffice to account for the behavior of excised rhubarb leaves subjected to culture under various conditions. Burkhart (25) has also expressed dissatisfaction with this hypothesis as an explanation of the behavior of the amides of seedlings.

On the other hand if amide synthesis is regarded as an expression of the availability respectively of oxaloacetic and α-ketoglutaric acids, these being supplied in the course of the respiration of the tissues, the wide variations in the behavior of different tissues, or of the same tissue under different circumstances, become intelligible.

The respiration of excised leaves in culture is by no means merely a matter of carbohydrate oxidation. This has been discussed in another paper (26) in which it is shown that a wide variety of the components of rhubarb leaves may be drawn upon for the carbon dioxide that is eliminated. Although it seems safe to assume that the soluble carbohydrates are earliest involved and probably do furnish a considerable part of the carbon lost, this depends to a large extent on how rich the leaves were in such components at the start. Even the residues from the deamination of amino acids produced by hydrolysis of the proteins may become involved, and it was possible to demonstrate this clearly in the case of rhubarb leaves that were initially somewhat low in soluble carbohydrates. Furthermore even with rhubarb leaves comparatively rich in carbohydrates, it was shown that a part of the carbon eliminated in the course of respiration was derived from soluble and insoluble components that were not reducing substances, and also that some of the load was borne by the organic acids, particularly by the malic acid in the petioles.

Thus repiration may in certain circumstances draw heavily upon many types of leaf components including the proteins. If the Krebs citric acid cycle be regarded as a model of the types of reaction that may take place, and the modifications of Chibnall are included which show how fat and protein components may enter the cycle after oxidation to such substances as succinic acid or α-keto acids, a mechanism is obtained which provides for a supply of the two substances fundamental to the synthesis of asparagine and glutamine, namely oxaloacetic acid and α-ketoglutaric acid. These may accordingly be derived in many different ways, and whether either or both arise is clearly a matter of the initial composition of the plant material, of the specific nature of its metabolism, and of the conditions under which it is studied.

It remains to be seen whether this view is in accordance with the observed behavior of plant tissues. The critical experiment of infiltrating leaf tissue with the ammonium salt of α-ketoglutaric acid and demonstrating the simultaneous decrease of this substance and increase of glutamine has been successfully performed by Chibnall and his associates (15). The evidence was not absolutely conclusive since respiration of the tissue during the experimental period brought about a loss of carbohydrates and these may conceivably have provided the carbon chain of the glutamine; this, however, seems highly improbable.

The classical example of amide synthesis in seedlings is difficult to discuss, since there are few data in the literature that have been obtained with due consideration of the properties of glutamine. If methods of extraction with hot solvents are employed, or if ammonia determinations are made by the technique of aeration from strongly alkaline solutions, more or less decomposition of this substance inevitably occurs and thus artificially enhanced values for ammonia nitrogen and depressed values for amide nitrogen are obtained. An example that may be due to this is to be found in Burkhart's data (25) which show a greater relative enrichment in ammonia in the case of pumpkin seedlings (known to contain glutamine (4)) than in yellow lupine seedlings which are mainly asparagine-forming plants. Nevertheless Burkhart's demonstrations that amides increase and that ammonia derived from the culture solution is utilized during the period when ample reserves of protein, fat, or carbohydrate were still present while, in the later periods when the stores of fat or carbohydrate had become depleted, ammonia accumulates and even the amides themselves may be partly decomposed are in agreement with the view that the changes, in so far as they affect the amides, are a reflection of the chemical nature of the substances respired and accordingly of the supply of the related α-keto acids.

Examples of the behavior of leaf tissues are to be found in previous papers from this laboratory. Tobacco leaves (8, 21) subjected to culture in darkness underwent a marked enrichment in asparagine while only moderate amounts of glutamine were formed. The protein was rapidly hydrolyzed and it has been shown that the nitrogen that contributed to the synthesis of the amides had its origin mainly in the amino nitrogen of the amino acids that were decomposed. For about 100 hours there was no significant increase in ammonia but subsequently it accumulated fairly rapidly.

When the leaves were cultured in light, both glutamine and asparagine were formed and, even after more than 200 hours, the increase in ammonia was small. The protein was hydrolyzed as promptly as in darkness but less completely. The amide metabolism in the two cases was thus strikingly different; in darkness only the precursor of asparagine was present, in light the precursors of both amides were formed and it seems evident that α-ketoglutaric acid was rendered available only as a result of the metabolism of the products of photosynthesis. The respiration in the two cases was doubtless widely different. In darkness only components originally present were available and, as the culture continued, the nature of the substances drawn upon must differ greatly if tobacco leaves behave in a manner at all similar to rhubarb leaves. It would seem, however, that the path followed was one that rendered only oxaloacetic acid available in significant amounts for reaction with ammonia. In light, on the other hand, the products of photosynthesis were present and the conversions that these underwent in the process of respiration were apparently such that α-ketoglutaric acid was also made available.

In rhubarb leaves cultured either in light or in darkness, glutamine alone is formed (22). The actual losses of carbon have been studied with this material (26) and the respiration in both conditions of culture has been shown to draw upon a wide assortment of components. Nevertheless it would seem in this case that the mechanism is such that little or no oxaloacetic acid becomes available at any time for reaction with ammonia. On the other hand α-ketoglutaric acid is made available, but not necessarily at all stages of the culture. In one case the data indicated that a small amount of glutamine was synthesized within the 1st day or two but, subsequently, much of the glutamine formed appeared to have arisen directly from the hydrolysis of the protein. Nevertheless in the later phases of this experiment, when the protein digestion and amino acid deamination reactions were proceeding with great intensity, glutamine was again synthesized from other sources and indeed very rapidly. In this case the leaves were initially comparatively rich in carbohydrates. In another case, with leaves initially poor in carbohydrates, little if any glutamine was formed, in addition to that which may have been directly liberated from the protein, until the later phases of the culture. In these leaves respiration was found to draw upon an even wider range of the initial components than in the other series and, in both, ammonia was present in very high concentration throughout. Obviously glutamine synthesis was not stimulated at all by this condition but only occurred when the proper nitrogen-free precursor became available in adequate amounts. This is unintelligible on the basis of the detoxication hypothesis but is readily understood if it be assumed that α-ketoglutaric acid is produced or, at least, made available for reaction with ammonia only at certain stages through the respiratory activity of the tissues.

It is important to note that the view that amide metabolism is connected with respiration is by no means novel. It was suggested as a possibility by Boussingault in 1864 (27) and has been considered by several subsequent investigators. Prianischnikow in particular (28) was convinced in 1899 that the nitrogen atom that formed the amide group must have arisen from the oxidation of an amino acid produced by hydrolysis of the protein. In later papers (7), however, save for the statement that ammonia is produced by oxidation reactions and that the carbon chain of the amides arises from the metabolism of carbohydrates, he made no further attempt to account for the detailed mechanism of amide synthesis.

EXPERIMENTAL

Most of the data from which the conclusions discussed above have been drawn have been published in previous papers from this laboratory (8, 23), the present interpretation being a development of views based largely on Chibnall's recent general discussion of protein metabolism in plants (15). The demonstration that tobacco plants can be produced which contain extraordinarily high concentrations of ammonia but which do not show abnormally high concentrations of amides or any marked effect upon the reaction of the tissues was made by growing plants (Rosenberg strain, one per pot) in sand culture with continuous renewal of nutrient solution. The solutions were made by dilution of stock solutions so that the composition of the one that contained all of its nitrogen as nitrate was KH_2PO_4 0.001125 M, K_2HPO_4 0.001125 M, $MgSO_4$ 0.0025 M, $Ca(NO_3)_2$ 0.0045 M. To vary the composition with respect to the form of nitrogen, the molarity of the calcium nitrate was reduced by steps and replaced with an equivalent of ammonium sulfate; in addition calcium chloride equivalent to the ammonium sulfate was also added to preserve the calcium level unchanged. To each solution, on being diluted to final volume, was added sufficient of stock solutions of salts of boron, manganese, and iron to provide 0.25 to 0.5 part per million of each of these elements. The reactions of the nutrient solutions were in the range pH 5.8 to 6.0 and they were supplied to the plants at the rate of about 800 ml. per day as the plants matured. The pH of the effluent seldom dropped below 4.5 and was usually above 5.0.

TABLE I

Composition of Tobacco Leaves, at Time of First Flower-Bud Formation, from Plants Grown in Sand Culture Supplied with Complete Nutrient Solution at Fixed Nitrogen and Calcium Levels but with Variation in Relative Proportion of Nitrate and Ammonia Nitrogen

Figures not otherwise designated are gm. per whole plant.

Percentage of N of nutrient solution as ammonia N	0	20	40	60	80	90
Organic solids	32.3	39.7	29.2	29.2	21.4	19.6
Total N	2.42	2.98	2.30	2.23	1.74	1.58
Organic N (total N − ammonia N − nitrate N)	1.81	2.29	1.74	1.87	1.46	1.34
Soluble organic N	0.521	0.650	0.466	0.570	0.497	0.493
Ammonia N	0.012	0.019	0.019	0.065	0.180	0.169
Amide N	0.027	0.034	0.030	0.035	0.046	0.051
Ammonia N as % of soluble organic N + ammonia N	2.25	2.84	3.92	10.3	26.6	25.5
Amide N as % of soluble organic N + ammonia N	5.06	5.08	6.19	5.51	6.79	7.70

The plants were harvested at the time of initial flower-bud formation, four to six individuals in each group. They were dissected into leaf, stalk, and root fractions and the tissues were dried at 80° for analysis. The data for the leaves only are given in Table I. The composition of the leaves of the plants grown on nitrate alone conforms quite closely with that of normal leaves from field-grown plants previously examined. The data for organic solids show that the plants with 20 per cent of their nitrogen as ammonia were somewhat larger than the nitrate controls, but at higher levels were only slightly smaller until the 80 per cent proportion of ammonia was reached. The data for total nitrogen include both nitrate and ammonia nitrogen but these are deducted to give the "organic nitrogen" and the "soluble organic nitrogen." The percentages of ammonia and of amide nitrogen are calculated on the basis of the soluble organic nitrogen plus the ammonia nitrogen in order to conform with the method of calculation usually adopted in expressing such data.

SUMMARY

Explanations that have been advanced for the formation under certain circumstances of the amides asparagine and glutamine in plants have been discussed, and the ammonia detoxication hypothesis of Prianischnikow has been found inadequate to account for the behavior of the amides in ammonia-rich rhubarb leaves or in tobacco leaves grown under conditions that gave rise to a high concentration of ammonia.

Chibnall has recently suggested that the Krebs citric acid cycle hypothesis proposed to account for the respiration of carbohydrates in animals may be applied to the plant. This scheme of reactions provides means whereby oxaloacetic acid and α ketoglutaric acid, the most probable precursors respectively of asparagine and glutamine, may arise from several sources.

On this view amide metabolism becomes a phase of the general respiratory activity of the tissues and undue emphasis on ammonia as a stimulant to the reaction is removed. It is shown that the behavior of the amides in leaf tissues cultured under various conditions is in agreement with this conception, the variations in the nature and rate of formation of the amides being explicable in terms of variations in the details of the respiratory mechanism.

BIBLIOGRAPHY

1. Vauquelin and Robiquet, *Ann. chim.*, **57**, 88 (1806).
2. Piria, R., *Ann. chim. et physiq.*, series 3, **22**, 160 (1848).
3. Schulze, E., and Bosshard, E., *Landw. Versuch.-Stat.*, **29**, 295 (1883).
4. Schulze, E., *Z. physiol. Chem.*, **24**, 18 (1898).
5. Prianischnikow, D., and Schulow, P., *Ber. bot. Ges.*, **28**, 253 (1910).
6. Prianischnikow, D., *Ber. bot. Ges.*, **40**, 242 (1922).
7. Prianischnikow, D., *Biochem. Z.*, **150**, 407 (1924).
8. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agric. Exp. Stat., Bull. 399* (1937).

9. Suzuki, U., *Bull. Coll. Agric. Imp. Univ. Tokyo*, **2**, 409 (1897).
10. Beyer, A., *Landw. Versuch.-Stat.*, **9**, 186 (1867). Müller, C. O., *Landw. Versuch.-Stat.*, **33**, 311 (1887). Schulze, E., *Landw. Jahrb.*, **21**, 105 (1892).
11. Mothes, K., *Z. wissensch. Biol., Abt. E, Planta*, **19**, 117 (1933).
12. Schwab, G., *Z. wissensch. Biol., Abt. E, Planta*, **24**, 160 (1935).
13. Schwab, G., *Z. wissensch. Biol., Abt. E, Planta*, **25**, 579 (1936).
14. Knoop, F., and Oesterlin, H., *Z. physiol. Chem.*, **148**, 294 (1925).
15. Chibnall, A. C., Protein metabolism in the plant, New Haven (1939).
16. Krebs, H. A., and Johnson, W. A., *Enzymologia*, **4**, 148 (1937).
17. Knoop, F., and Martius, C., *Z. physiol. Chem.*, **242**, I (1936).
18. Martius, C., and Knoop, F., *Z. physiol. Chem.*, **246**, I (1937). Martius, C., *Z. physiol. Chem.*, **247**, 104 (1937); **257**, 29 (1938).
19. Annau, E., Banga, I., Blaszó, A., Bruckner, V., Laki, K., Straub, F. B., and Szent-Györgyi, A., *Z. physiol. Chem.*, **244**, 105 (1936).
20. Mothes, K., *Z. wissensch. Biol., Abt. E, Planta*, **1**, 472 (1926); **7**, 585 (1929).
21. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, **119**, 369 (1937).
22. Vickery, H. B., Pucher, G. W., Leavenworth, C. S., and Wakeman, A. J., *J. Biol. Chem.*, **125**, 527 (1938).
23. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agric. Exp. Stat., Bull. 424* (1939).
24. Ruhland, W., and Wetzel, K., *Z. wissensch. Biol., Abt. E, Planta*, **3**, 765 (1927); **7**, 503 (1929).
25. Burkhart, L., *Plant Physiol.*, **13**, 265 (1938).
26. Vickery, H. B., and Pucher, G. W., *J. Biol. Chem.*, **128**, 685 (1939).
27. Boussingault, J. B., *Compt. rend. Acad.*, **58**, 917 (1864).
28. Prianischnikow, D., *Landw. Versuch.-Stat.*, **52**, 347 (1899).

THE ERGOT ALKALOIDS

XVII. THE DIMETHYLINDOLE FROM DIHYDROLYSERGIC ACID

By WALTER A. JACOBS and LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, March 13, 1939)

Among the volatile products formed on fusion of dihydrolysergic acid (1) an indole fraction was obtained from which a picrate was isolated in very small amount which melted at 148–150°. The analytical figures obtained with it approximated those of the picrate of an indole $C_{11}H_{13}N$. The indole still gave the dimethylaminobenzaldehyde test and therefore presumably had the α (or β) position still free (2). It was not identical with a β-*n*-propylindole. Since it was formed in such small amounts and was very difficult to purify, we have turned again more recently to the previously reported indole acid contained in the non-volatile alkali melt, which was also formed in very small amount (1).

This indole acid fraction was again prepared and subjected to low pressure distillation during which decarboxylation occurred. The resulting indole after recrystallization was finally obtained in very small yield with a melting point of 115–117°. A picrate prepared from one of the fractions melted at 185–187°. Since there was not sufficient of either the indole itself or its picrate with these melting points for analysis, analyses were obtained with several picrate fractions of somewhat lower melting points (175–183° and 164–168°). These analyses were in fair agreement with the figures required by a dimethylindole. Since the indole still gave the dimethylaminobenzaldehyde reaction, it appeared probable that it could be a 3,4-dimethylindole resulting by decarboxylation of a 4-methylindole-3-acetic acid. This would be expected as a scission product from the structure which we have derived for lysergic acid.

As a check on this conclusion the indoles were prepared from *m*-tolylhydrazine and propionaldehyde. Mendlik and Wibaut (3) had previously attempted the same synthesis and obtained but one substance (m.p. 116–117° and picrate m.p. 179–180°) which they concluded to be 3,6-dimethylindole. However, we have obtained two isomers, as would be expected,[1] one which must be *3,4-dimethylindole* and the other *3,6-dimethylindole.* The identity of these has not been directly established except by inference from the comparison with the above indole from lysergic acid. One of these, which melted at 117–118°, gave a picrate melting at 182–183° and was thus identical with that described by Mendlik and Wibaut. Neither this indole nor its picrate gave a depression with the substances obtained from lysergic acid and agreed in other properties with these substances. It may therefore be inferred that this substance is 3,4-dimethylindole and not the 3,6 derivative. The formation of a 3,6 derivative, barring unlikely rearrangements, is incompatible with the structure that we have derived for lysergic acid and which is at the same time a derivative of naphthalene, quinoline, and indole. The second synthetic indole which melted at 90–93° and gave a picrate melting at 163–164° would therefore be 3,6-dimethylindole.

The attempt will be made, when opportunity presents, to confirm this by the synthesis of 3,4-dimethylindole by another method.

EXPERIMENTAL

3,4- and 3,6-Dimethylindoles—5 gm. of propionaldehyde were treated with 10.4 gm. of *m*-tolylhydrazine and warmed. The hydrazone was extracted with ether and the solution was dried over K_2CO_3. After concentration the residue was treated with 30 gm. of anhydrous zinc chloride and the mixture was heated in an oil bath raised to 165°. When the reaction was completed, the contents of the flask were washed out with ether and excess 10 per cent HCl. The washed and dried ether extract was concentrated in an apparatus for sublimation of the residue. All the material subliming up to an oil bath temperature of 125° and under 0.2 mm. pressure was collected and the residue discarded. The sublimate which weighed about 3 gm. was dissolved in 30 cc. of warm petroleum ether. When chilled to −15°, crystallization began. The crystals were filtered off without washing and the filtrate was set aside to be treated as described below.

---

[1] The formation of two isomeric oxindoles from the *m*-tolylhydrazide of propionic acid has already been reported (4).

The crystalline fraction after repeated crystallization from petroleum ether formed flat needles which melted at 117–118°.

$C_{10}H_{11}N$. Calculated, C 82.76, H 7.64; found, C 82.89, H 7.69

This indole yielded a picrate from ethyl alcohol forming dark red needles which melted at 182–183°.

$C_{16}H_{14}O_7N_4$. Calculated, C 51.34, H 3.74; found, C 51.37, H 3.78

The above petroleum ether filtrate from the crystalline indole was concentrated and transferred to a 22 cm. microfractionating column. Fractionation was carried out under approximately 0.2 mm. pressure. Five fractions were collected. Each fraction was about 0.15 gm. with the exception of the second which weighed 0.33 gm.

The second fraction completely solidified on the condenser and showed a melting point of 60–90°. When dissolved in 3 cc. of petroleum ether and chilled to −15°, crystallization began. 0.28 gm. of broad, thin leaflets was collected, which melted at 90–93°.

0.1 gm. was converted to the picrate in 3 cc. of ethyl alcohol. 0.175 gm. of needles was collected which melted at 163–164°.

$C_{16}H_{14}O_7N_4$. Calculated, C 51.34, H 3.74; found, C 51.45, H 3.97

The indole recovered from the picrate after redistillation melted at 90–92°.

$C_{10}H_{11}N$. Calculated, C 82.76, H 7.64; found, C 82.97, H 7.65

Dimethylindole from Dihydrolysergic Acid—1.2 gm. of dihydrolysergic acid were ground with 7 gm. of KOH and placed in a fusion apparatus as previously described (1). The salt bath for heating the mixture was held at 300° for 0.5 hour.

The volatile material from the reaction was worked up in the manner previously given and 10 mg. of the indole fraction were obtained. Since this proved difficult to purify further, attention was turned to the alkali melt.

This melt was dissolved in water and extracted with ether. The latter was discarded. The aqueous layer was then acidified to

Congo red with HCl and extracted repeatedly with ether. This extract was concentrated in a molecular still for distillation of the residue. 135 mg. of distillate were collected up to an oil bath temperature of 200° and under a pressure less than 0.001 mm. The distillate was dissolved in ether and extracted with NaOH solution. The alkaline extract was then acidified with HCl. The acid material which separated was reextracted with ether. Since it could not be directly crystallized, it was redistilled in a microstill under 0.5 mm. pressure with the temperature of the oil bath raised to 250°. During this procedure, the production of an indole by decarboxylation was obvious from the odor of the distillate. 87 mg. of material were collected. This was dissolved in ether and shaken out with NaOH solution to remove undecomposed indole acids. The ether layer upon evaporation gave an oily indole fraction which was redistilled in a microstill. 7 mg. distilled up to 150° under 0.3 mm. pressure. The product melted at 80–100°. Upon recrystallization from 0.02 cc. of petroleum ether, 3 mg. of flat needles were obtained which melted at 108–112°.

When this was treated with an equivalent of picric acid in 0.15 cc. of ethyl alcohol, 3.2 mg. of red needles were obtained which melted at 182–186°. After recrystallization from alcohol the substance melted at 185–187°. A mixed melting point with the higher melting synthetic picrate obtained above showed no depression.

The final picrate was recombined with its mother liquor and evaporated to dryness. The residue (3.2 mg.) was treated with a small volume of sodium hydroxide and a few drops of ether. The ether layer was washed with NaOH solution until all color of picric acid had disappeared and then evaporated to dryness in a microstill for redistillation of the indole. 1.5 mg. distilled up to 150° and under 0.3 mm. This material melted at 115–116°. After recrystallization from 0.01 cc. of petroleum ether, 0.8 mg. of long flat needles was obtained which melted at 115–117°. A mixed melting point with the higher melting synthetic indole reported above showed no depression.

Since the supply of the pure, high melting indole from dihydrolysergic acid was exhausted in melting point determinations, there was not sufficient for analysis as the picrate or the indole itself.

Analytical data were obtained on a lower melting picrate (164–168°) obtained in a preliminary run as follows:

$C_{16}H_{14}O_7N_4$. Calculated, C 51.34, H 3.74; found, C 51.70, H 3.92

From the mother liquors of the purified indole, 2.2 mg. of indole were recovered which melted at 80–105°. A picrate (3 mg.) prepared from this melted at 175–183°. This was not recrystallized, since there was not enough for both recrystallization and analysis. The analysis gave C 51.90, H 4.06.

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **111**, 455 (1935).
2. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **113**, 767 (1936).
3. Mendlik, F., and Wibaut, J. P., *Rec. trav. chim. Pays-Bas*, **50**, 91 (1930).
4. German patents No. 218,477 and No. 218,727; Friedländer, P., Fortschritte der Teerfarbenfabrikation und verwandter Industriezweige, Berlin, **9**, 967 (1911).

CHOLESTEROL CONTENT OF SKIN, BLOOD, AND TUMOR TISSUE IN RATS IRRADIATED WITH ULTRAVIOLET LIGHT*

By ARTHUR KNUDSON, STUART STURGES, and W. RAY BRYAN

(From the Departments of Biochemistry and Pathology, Medical Department of Union University, Albany Medical College; Albany)

(Received for publication, March 27, 1939)

The production of malignant tumors by ultraviolet radiation has been reported by several investigators. Herlitz, Lundill, and Wahlgren (1), Findlay (2), and Putschar and Holtz (3) have produced malignant growths in mice and rats by irradiation. Roffo (4) reported an extensive series of experiments in which he exposed rats to ultraviolet radiation, beginning with a 5 minute daily dose, and gradually increasing to 20 hours daily. Tumors developed on the eyes, ears, and back of the head in 6 to 8 months. Beard, Boggess, and von Haam (5), applying the technique of Roffo, have verified these observations, producing sarcomas and carcinomas on the eyes, ears, and heads of rats.

In the field of tumor chemistry, cholesterol has been of particular interest. Kahn (6) showed that malignant tumors contain large amounts of cholesterol and its esters, while blood serum was lower than normal in cholesterol. Bolaffi (7) reported an increase of cholesterol esters in the whole organism of tumor mice. Bergheim (8), using histological methods, found larger amounts of cholesterol in malignant tumors than in benign ones. Yasuda and Bloor (9) likewise report that malignant tumors contain a much higher percentage of fatty substances—neutral fat, phospholipid, and cholesterol—than less malignant ones. Roffo (10–12) has emphasized that cholesterol must play a predominant part in the formation of tumors. This is based on his observations that cholesterol is greatly increased in neoplastic tissue, particularly in tumors of the skin, that it is present in precancerous stages, and that exposure of rats to sunlight and ultraviolet radiation results in great increases in the cholesterol content of the skin. He suggests that cholesterol prepares the soil for subsequent malignant growth by acting as an accumulator of light.

---

* Presented before the annual meeting of the American Society of Biological Chemists at Baltimore, March, 1938.

EXPERIMENTAL

Using similar technique and lamps as Beard, Boggess, and von Haam (5), we exposed adult rats of both albino and piebald strains to ultraviolet radiation. During the period of exposure they were fed on our stock diet of Wayne dog food, Blox.[1] The rats were kept in galvanized wire cages containing compartments 6 × 8 × 8 inches, which were large enough for either one or two rats. The lamps were suspended from the ceiling so that they were 4 feet from the bottoms of the cages and centered directly over the cages. After the dose was increased gradually to 20 hours per day, this dose was then continued daily.

During the period of irradiation, some of the animals died apparently from excessive heat, and the skin tissues of these were taken for analysis. Other animals were killed off at varying intervals after irradiation, and skin tissues and blood analyzed for total and free cholesterol. The skin tissue from the face, back, and abdomen was analyzed for total and free cholesterol according to the method of Sturges and Knudson (13). The blood cholesterols were determined by the Schoenheimer and Sperry (14) method.

After 2 months of irradiation, skin lesions began to appear and the first tumors occurred in about 5 months. Later, other tumors appeared. These tumors developed on the eyes, ears, and nose, and were of the same type as those described by both Roffo (4) and Beard, Boggess, and von Haam (5). The animals with tumors were killed off at various intervals and the tumors and the skin analyzed for total and free cholesterol. The tumor tissue was carefully cleaned, and, if necrotic, this was separated from the rest of the tissue and analyzed separately.

DISCUSSION

The results of the analyses for total and free cholesterol on the skin from the face, back, and abdomen of irradiated rats and of non-irradiated rats are given in Table I. The irradiated groups of animals are divided into those without tumors and those with tumors.

---

[1] Manufactured by the Allied Mills, Inc., Chicago.

It will be noted that in the irradiated rats the total cholesterol of the skin and particularly that from the face is greatly increased. The free cholesterol is increased but not to as great an extent. In the group of irradiated rats with no tumors, which were exposed from 230 to 3570 hours, the increases in cholesterol do not run parallel with the length of radiation. The average total cholesterol in the skin from the face for this group is increased almost 100 per cent, while in one animal it is over 170 per cent above the normal average. These increases of total cholesterol in the skin after ultraviolet irradiation are in agreement with the results reported by Roffo (11). It is rather significant that the increase in cholesterol should be largely in the ester form, so that the average percentage of free cholesterol drops from 55.2 per cent to 31.2 per cent. The cholesterol content of the skin from the back is also increased but much less. The average increase is only about 34 per cent and there is no change in the relationship of free to total cholesterol. In the skin from the abdomen there is relatively little difference in the irradiated animals without tumors and the controls.

In the group of animals that have developed tumors, the cholesterol content of the skin is found to be still further increased. These animals were irradiated from 3110 to 4950 hours over a period of 167 to 259 days. The total cholesterol in the skin from the face of these rats is on the average 150 per cent above normal. The cholesterol content of the skin from the back and the abdomen in this group of rats is also increased but to a less extent. In those rats showing the greater increase in total cholesterol in the skin from both the back and the abdomen it is to be noted again that the increases are largely in the ester form. Regarding the significance of the great increase in cholesterol in the skin after ultraviolet irradiation, little can be said. That the increase in cholesterol should be largely in the ester form is extremely interesting. Does the ester form fix the cholesterol more firmly in the tissue? Is the increase in cholesterol a predominant factor and a cause for the development of the tumor which follows long continued ultraviolet irradiation? These are questions which must await further study before definite answers can be given.

In Table II are given the results of analyses for total and free cholesterol on tumors produced by ultraviolet irradiation. The total cholesterol content in most of the tumors is high compared to the cholesterol content of normal skin from the face of rats. There is apparently no relationship with respect to the cholesterol

TABLE I

Cholesterol Content of Skin Tissues of Rats Exposed to Ultraviolet Radiation

The results are given in mg. per 100 gm. of dry tissue.

Rat No.	Period of radiation		Face			Back			Abdomen		
			Total	Free	Free/Total	Total	Free	Free/Total	Total	Free	Free/Total
						Normals					
	days	*hrs.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
9698			314	250	79.6	506	229	45.2	550	333	60.6
9879			595	383	64.4	653	164	25.1	580	252	43.4
1			652	350	53.7	458	144	31.4	652	350	53.7
2			559	246	44.0	219	97	44.3	310	144	46.5
3			664	368	55.4	268	102	38.1	391	175	44.8
4			461	194	42.1	271	95	35.1	336	114	33.9
Average....			541	299	55.2	396	139	35.1	470	228	48.7
						Without tumor					
198	23	230	1240	437	35.2	1095	518	47.2	544	204	37.5
220	23	230	1300	435	33.4				645	214	33.2
77	33	430	1100	327	29.7	521	246	47.2	520	231	44.4
78	33	430	496	264	53.2				374	183	48.9
103	33	430	1205	316	26.2	630	244	38.7	784	312	39.8
75	54	850	958	358	37.4	547	223	40.8	390	173	44.3
76	54	850	959	363	37.9	575	225	39.2	634	308	48.6
191	86	1490	1170	396	33.8	785	268	34.2	649	189	29.1
23	91	1590	1100	210	19.1	505	137	27.2	493	294	59.6
20	92	1610	1330	234	17.7	356	134	37.6	310	108	34.8
9692	150	2770	655	226	34.5	699	204	29.2	381	193	40.1
9703	150	2770	786	189	24.0	448	154	34.4	461	154	33.4
9737	150	2770	1190	369	31.0	817	264	32.3	630	270	42.8
216	162	3010	976	294	30.1	562	221	39.3	390	257	66.0
204	172	3210	1570	510	32.5	925	276	29.8	904	364	40.3
214	190	3570	730	339	46.5	452	194	42.9	634	308	48.6
Average...			1048	327	31.2	637	236	37.1	553	235	42.6

TABLE I—*Concluded*

Rat No.	Period of radiation		Face			Back			Abdomen		
			Total	Free	Free/Total	Total	Free	Free/Total	Total	Free	Free/Total
With tumor											
	days	*hrs.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
417	167	3110	2305	535	23.2	1139	270	23.7	626	294	47.0
232	168	3130	990	250	25.3				571	179	31.4
219	168	3130	694	395	56.9	620	184	29.7	353	196	55.5
202	168	3130	1453	419	28.8	608	194	32.0	780	286	36.7
413	174	3250	1055	162	15.4	697	152	21.7	324	100	30.8
422	186	3490	916	270	29.4	2340	598	25.5	732	304	41.5
197	189	3550	1108	425	38.4	523	302	57.6	388	360	92.9
222	211	3970	1620	516	31.9	675	219	32.4	585	361	61.8
188	221	4170	1225	272	22.2	517	127	24.5	310	146	47.1
231	232	4410	1755	546	31.1	1015	343	33.8	1112	556	50.0
190	248	4670	2118	530	25.1	578	195	33.8	1220	340	27.9
189	248	4730	1159	394	34.0	1171	273	25.9	541	224	41.4
187	259	4910	1490	419	28.1	575	230	40.0	406	233	57.4
211	259	4950	1248	378	30.2	862	223	25.9	930	316	34.0
223	259	4950	1680	1150	68.4	1090	183	16.8	714	268	37.6
Average . . .			1388	444	32.0	886	250	28.2	639	278	43.5

TABLE II

Cholesterol Content of Tumors Produced by Ultraviolet Radiation

The results are given in mg. per 100 gm. of dry tissue.

Rat. No.	No. of days radiation	Total hrs. radiation	Dry substance	Cholesterol		
				Free	Total	Free/Total
			per cent	*mg.*	*mg.*	*per cent*
417	167	3110	32.3	1125	1360	82.8
422	186	3490	18.9	895	984	91.0
222	211	3970	14.4	939	1075	87.5
188	221	4170	48.8	293	439	69.0
231	232	4410	17.7	1285	1427	90.1
225	238	4530	17.8	1075	1328	81.0
190	245	4670	19.2	582	697	83.5
189*	248	4730	26.8	1193	1330	89.7
189*	248	4730	18.8	659	732	89.8
203*	248	4730	70.7	906	1450	62.5
203*	248	4730	28.5	1210	2770	47.3
187	257	4910	59.2	262	302	86.8
211	259	4950	45.5	412	542	76.0
223	259	4950	19.3	957	1005	95.5
Average				842	1031	80.9

* Two tumors from each animal, right and left ears respectively.

content of the tumors and the length of irradiation. The free cholesterol content of the tumors averages approximately 81 per cent, while the free cholesterol content of the skin from the face of irradiated rats averages only about 33 per cent.

TABLE III

Cholesterol Content of Blood of Rats with Tumors Produced by Ultraviolet Radiation

The results are given in mg. per 100 gm. of blood.

Rat No.	Period of radiation		Total	Free	$\frac{\text{Free}}{\text{Total}}$
			Normals		
	days	*hrs.*	*mg.*	*mg.*	*per cent*
1			69.0	21.5	31.2
2			81.7	23.0	28.2
3			70.9	20.0	27.2
4			69.0	16.6	24.1
Average...			72.7	20.3	27.9
			Irradiated		
417	167	3110	39.2	9.8	25.0
422	186	3490	42.0	30.9	76.3
231	232	4410	41.0	17.1	41.0
225	238	4530	33.3	15.6	46.8
190	248	4670	29.8	13.5	45.3
189	248	4730	36.4	13.9	32.2
203	248	4730	54.0	41.6	77.0
187	259	4910	16.7	8.8	52.7
211	259	4950	33.7	12.1	35.9
223	259	4950	26.9	8.2	30.5
9689	259	4950	38.6	25.6	66.3
Average..			35.7	17.9	50.1

There is, then, a significant difference in the relationship of free to total cholesterol content in the tumors produced by radiation compared to that in the skin of irradiated rats.

A few determinations on the total and free cholesterol content of the blood of irradiated rats with tumors are given in Table III. The total cholesterol content is somewhat below the normal level, which is in striking contrast to the increases of cholesterol noted in both the skin and tumors of irradiated animals.

SUMMARY

The total cholesterol content of the skin is increased by ultraviolet irradiation. This is particularly true in the skin from the face which showed increases in some instances of several hundred per cent, and averaged about 100 per cent. The cholesterol content of the skin from the back and abdomen is only slightly increased. The increases of total cholesterol in the skin are practically all in the ester form. There is no relationship between the increases of total cholesterol and the period of irradiation.

The tumors formed by ultraviolet irradiation show a high cholesterol content and about 80 per cent is in the free form.

The total cholesterol content of the blood of irradiated rats with tumors is somewhat below the normal level.

Addendum—After this paper was submitted for publication, Baumann and Rusch (15) reported that the cholesterol content of the skin was increased by irradiation in the rat, but not in the mouse or in the guinea pig. The increase in cholesterol content of rat skin was common to both the exposed and protected portions.

BIBLIOGRAPHY

1. Herlitz, C. W., Lundill, I., and Wahlgren, F., *Acta pædiat.*, **12**, 221 (1932).
2. Findlay, G. M., *Lancet*, **1**, 1229 (1930).
3. Putschar, W., and Holtz, F., *Z. Krebsforsch.*, **33**, 219 (1931).
4. Roffo, A. H., *Bol. Inst. med. exp. estud. y trat. cancer*, **10**, 417 (1933).
5. Beard, H. H., Boggess, T. S., and von Haam, E., *Am. J. Cancer*, **27**, 257 (1936).
6. Kahn, H., *Klin. Woch.*, **4**, 178 (1928).
7. Bolaffi, A., *Z. Krebsforsch.*, **30**, 170 (1929–30).
8. Bergheim, F., *Klin. Woch.*, **13**, 828 (1929).
9. Yasuda, M., and Bloor, W. R., *J. Clin. Inv.*, **11**, 677 (1932).
10. Roffo, A. H., *An. Facult. med., Montevideo*, **1** (1916).
11. Roffo, A. H., *Am. J. Cancer*, **17**, 42 (1933).
12. Roffo, A. H., and Pilar, F., *Bol. Inst. med. exp. estud. y trat. cancer*, **7**, 22 (1930).
13. Sturges, S., and Knudson, A., *J. Biol. Chem.*, **126**, 543 (1938).
14. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
15. Baumann, C. A., and Rusch, H. P., *Am. J. Cancer*, **35**, 213 (1939).

THE ULTRACENTRIFUGAL ANALYSIS OF THE LATENT MOSAIC VIRUS PROTEIN

By RALPH W. G. WYCKOFF

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton)

(Received for publication, March 30, 1939)

The virus protein that is the infectious principle of the latent mosaic disease of potatoes was first isolated by differential ultracentrifugation (1, 2) more than 2 years ago. It is much less stable than the corresponding protein of the tobacco mosaic disease; nevertheless, if sufficient care is taken its purified solution will yield a sharp centrifugal pattern indicative of an undamaged molecular species. Preparations also have been made by chemical procedures (3). Their properties have been in some ways different from those of the proteins isolated by ultracentrifugation. For this reason it has been important to see whether simple chemical treatments would lead to molecular alterations detectable in the analytical ultracentrifuge. The present paper records the results of several such observations together with a detailed study of the sedimentation pattern of this virus protein at a variety of pH values.

The writer is indebted to H. S. Loring for the samples used in this work. Parallel studies (4) of the biological activities and of virus properties of these samples have recently been described by him. Quantity ultracentrifugations were made in the same apparatus (5) that has been employed in all ultracentrifugal preparations (6) of this and other plant virus proteins. This machine carries heads about 7 inches in diameter and holds 150 cc.; the sedimentations necessary for the ultracentrifugal purification of the latent mosaic virus protein were effected by running the machine for an hour and a quarter at a speed of about 30,000 R.P.M. The analytical observations were carried out by the original absorption technique of Svedberg. The optimum centrifugal field for such studies on the latent mosaic protein is about 30,000 times gravity.

For ultracentrifugal sedimentations the quantity heads were chilled in ice before use. The temperature of the head and its contained materials was never more than a few degrees above freezing at the conclusion of a run. Under such circumstances the virus protein of the latent potato disease can be repeatedly precipitated and resuspended in distilled water or dilute buffer solutions without suffering damage detectable with the analytical ultracentrifuge. The sedimentation diagram of a typical preparation obtained by subjecting the clarified juice of infected Turkish tobacco plants to four successive ultracentrifugations is shown in Fig. 1. The principal boundary is sharp; it moves at a rate corresponding to the constant $s_{20} = 113 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1} (Table I). A faint sharp second boundary with s_{20} = about 130×10^{-13} can also be seen; the component causing this is some-

TABLE I

Sedimentation Constants of Latent Mosaic Virus Protein Solutions

pH	s_{20}	Time in buffer before being photographed	Remarks
3.3	114, 129	Immediate	Diffuse boundary
3.3		1 day	Complete precipitation
3.8	114, 137	Immediate	Diffuse
3.8	113	1 hr. then readjusted	Sharp
3.8	124, 141	20 hrs.	Very diffuse
4.1		1 day	Complete precipitation
5.7	112, 127	Immediate	Sharp; one preparation shows double boundary, other does not
6.1	111, 127	"	" "
7.0	112, 126	"	" "
7.0	109	3 days	Sharp
8.1	107	Immediate	"
9.8	113	"	"
10.1	109	"	"
10.1	109	36 hrs.	" unsedimentable
10.1	107	7 days	" "
10.3	*Ca.* 75, 115	Immediate	Moderately diffuse
11.0	<3	"	

times absent, no matter whether the sample has been purified by ultracentrifugation or by salting-out.

Though repeated chemical precipitations lead to obvious damage of the virus protein, a single precipitation with either 30 per cent ammonium sulfate or potassium citrate followed by two ultracentrifugal sedimentations has given a sharp boundaried product if the salting-out was carefully carried out in the cold. Precipitation by HCl at pH 4 furnished a protein with a moderately sharp boundary. The protein precipitated by an equal volume of alcohol added at pH 5 was so thoroughly altered that it gave no measurable sedimenting boundary.

In previously reported investigations (7-9) sedimentation diagrams have been made of several virus protein solutions at a wide variety of pH values. These studies have demonstrated that in each instance the virus protein molecule and the infectivity of the virus have the same range of pH stability. This is to be expected if infectivity is a property of the intact virus protein molecule. The same type of study has now been made of the latent mosaic virus protein. Sedimentation diagrams have been prepared of solutions of the protein at pH values between 3.3 and 11 (Table I). Some of these diagrams have been obtained immediately after pH adjustment, others after standing for various lengths of time. These results should be compared with the pH inactivation studies of Loring (4). Such a comparison makes it clear that this virus protein molecule too is stable within the same general limits that define its virus activity. The same constant, within experimental error, is observed from all preparations at pH values between about 5.7 and 10.1. On the alkaline side of pH 10.1 the breakdown is practically immediate and complete, in contrast to the behavior of the tobacco mosaic virus protein. At pH 10.3 a more rapidly moving boundary with s_{20} = about 75×10^{-13} appears (Fig. 5); the boundaries of solutions standing at this pH rapidly became so diffuse that accurate measurements were impossible. Slightly greater alkalinity (pH 10.7 to 11.0) leaves fragments that sediment no faster than the molecules of egg albumin (Fig. 6). In acid affairs are not so simple, because the limit of stability is not far from the isoelectric point of the protein (at pH 4). In the range from about pH 4 to 5 not enough of the protein is in solution to provide a boundary. At pH 3.8

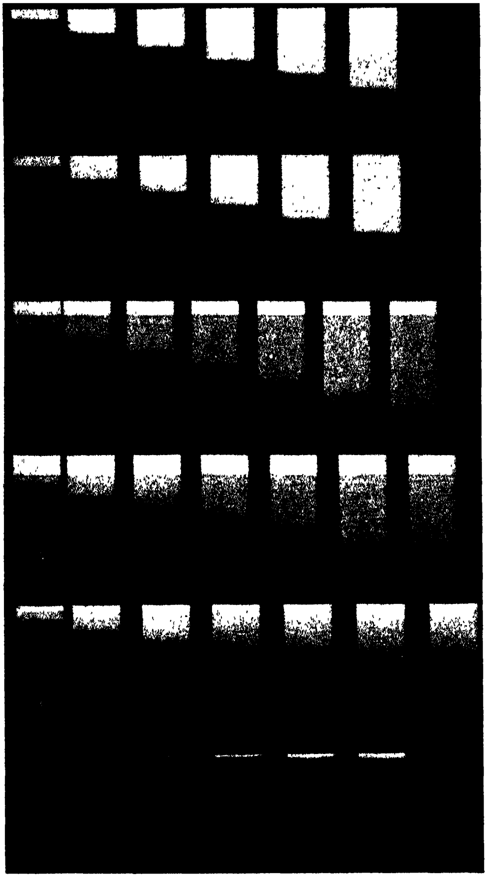


FIG. 1. Sedimentation diagram of a sample of the latent mosaic virus protein purified by a 4-fold quantity ultracentrifugation with distilled water as resuspending fluid. In this and each of the following diagrams the interval between exposures was exactly 5 minutes and the mean centrifugal field about 31,000 times gravity.

FIG. 2. Diagram of the virus protein in pH 5.0 buffer immediately after pH adjustment.

FIG. 3. Diagram of the virus protein made immediately after adjustment to pH 3.8.

FIG. 4. Diagram of the virus protein after being held for 1 hour at pH 3.8 and then readjusted to pH 7.

FIG. 5. Diagram of the virus protein made immediately after adjustment to pH 10.3.

FIG. 6. Diagram of the virus protein made immediately after adjustment to pH 11.0.

the usual boundaries can be photographed but they are only moderately sharp immediately after pH adjustment (Fig. 3) and a day later are very diffuse. That the molecular change responsible for this diffuseness is reversible and perhaps due to associations in the neighborhood of the isoelectric point is indicated by the fact that if the pH is readjusted to neutrality after a short time at pH 3.8, a sharp pattern is once more obtained (Fig. 4). Loring found (4) that such readjusted solutions had their full activities. A photograph made immediately after adjustment to pH 3.3 also shows a diffuse boundary but after a day at this acidity precipitation is complete and no boundary can be observed.

BIBLIOGRAPHY

1. Stanley, W. M., and Wyckoff, R. W. G., *Science*, **85**, 181 (1937).
2. Loring, H. S., and Wyckoff, R. W. G., *J. Biol. Chem.*, **121**, 225 (1937).
3. Bawden, F. C., and Pirie, N. W., *Brit. J. Exp. Path.*, **17**, 64 (1936); **19**, 66 (1938).
4. Loring, H. S., *J. Biol. Chem.*, **126**, 455 (1938).
5. Wyckoff, R. W. G., and Lagsdin, J. B., *Rev. Scient. Instruments*, **8**, 74 427 (1937).
6. Wyckoff, R. W. G., *Proc. Am. Phil. Soc.*, **77**, 455 (1937).
7. Wyckoff, R. W. G., *J. Biol. Chem.*, **122**, 239 (1937–38); **124**, 585 (1938).
8. Wyckoff, R. W. G., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, **36**, 562 (1937). Beard, J. W., and Wyckoff, R. W. G., *J. Biol. Chem.*, **123**, 461 (1938).
9. Beard, J. W., Finkelstein, H., and Wyckoff, R. W. G., *Science*, **86**, 331 (1937); *J. Immunol.*, **35**, 415 (1938).

RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

VII. THE INFLUENCE OF CHOLESTEROL UPON PHOSPHOLIPID TURNOVER IN THE LIVER *

BY I. PERLMAN AND I. L. CHAIKOFF

(From the Division of Physiology of the Medical School, University of California, Berkeley)

(Received for publication, March 27, 1939)

It has long been suspected that phospholipid formation is intimately connected with fat transfer, and in a previous report from this laboratory it was definitely established that an increased phospholipid turnover is associated with the action of choline in its sweeping out of fat from the liver of rats fed high fat diets (1). It has also been known for some time that fatty livers are readily produced by feeding rats diets rich in cholesterol (2–4) and that the excess lipid deposition in the liver under these conditions is prevented by choline (5, 6). The finding of an inverse relation between fat deposition in the liver and phospholipid exchange (as measured by radioactive phosphorus) during choline action (1) suggested that cholesterol might serve to depress such phospholipid exchange. The observations reported here demonstrate that this is indeed the case: The ingestion of cholesterol decreased the liver content of newly formed phospholipid which had been labeled by means of a radioactive isotope of phosphorus. The stimulating effect of choline on the depressed phospholipid metabolism found under these conditions is also presented.

EXPERIMENTAL

The results recorded here were obtained from separate analyses of 220 rats, the details of which are shown below. The experimental feedings were of short duration, about 30 hours, and before

---

* Aided by a grant from the Research Board of the University of California.

this the rats were maintained on an adequate stock diet. Two experimental diets were employed.

Control Diet—This consisted of 5 gm. of unsalted butter to which were added 0.015 cc. of a standardized cod liver oil and 0.1 cc. of a vitamin B concentrate.

Cholesterol Diet—This consisted of the above constituents and additions of cholesterol in amounts varying from 250 to 500 mg.

Radioactive phosphorus was injected subcutaneously as an isotonic solution of Na_2HPO_4. Choline in the form of its chloride was fed by stomach tube.

The livers were excised at various intervals after phosphorus injections. Their phospholipids were isolated in a manner previously described (7). The method by which the phospholipids were mounted and their radioactivity determined has been noted elsewhere (8).

Results

Effect of Short Feedings of Cholesterol upon Phospholipid Turnover in Liver—60 rats were fasted for 24 hours and then placed on the control and experimental diets described above. 26 hours after first receiving these diets, all rats were injected with labeled phosphate and their livers removed 4 hours after. The total period of feeding with cholesterol diet was therefore 30 hours. The phospholipid activity[1] of the liver in the rats that had received cholesterol as well as in those that had ingested the control diet is shown in Table I.

For consideration of the results, the experiments were divided into three series. In Series 1, rats weighing 160 to 185 gm. ingested an average of 440 mg. of cholesterol during a period of 30 hours. In their livers an average of 1.93 per cent of the administered phosphate was found as labeled phospholipid, as compared with the control level of 2.42 per cent.

In Series 2, eight rats that had received the butter-vitamin diet served as control for two other groups of eight rats that were fed cholesterol diets. The amounts of cholesterol ingested by these

---

[1] The term phospholipid activity refers to the percentage of administered labeled phosphorus found in the form of phospholipid. Throughout this study the activity of the whole liver is recorded. Animals were carefully paired according to weight and sex.

TABLE I

Effect of Cholesterol upon Level of Newly Formed Phospholipid in Liver

Series No.	Control		Cholesterol-fed		Cholesterol-fed	
	Rat No.	Activity*	Rat No.	Activity	Rat No.	Activity
1†	1	2.38	7	1.83		
	2	2.56	8	1.78		
	3	2.48	9	2.06		
	4	2.31	10	2.21		
	5	2.31	11	1.81		
	6	2.47	12	1.89		
Average activity		2.42		1.93		
" butter ingested, *gm.*		7.1		7.3		
" cholesterol ingested, *mg.*			440			
2‡	205	2.62	213	2.63	221	3.06
	206	3.82	214	2.94	222	2.21
	207	3.35	215	2.52	223	2.17
	208	2.84	216	2.21	224	2.35
	209	2.78	217	2.04	225	2.06
	210	2.76	218	2.30	226	2.34
	211	3.10	219	2.09	227	2.21
	212	2.83	220	3.25	228	2.07
Average activity		3.01		2.50		2.31
" butter ingested, *gm.*		9.7		10.9		9.6
" cholesterol ingested, *mg.*			545		963	
3§	229	3.94	237	2.40	245	2.95
	230	3.66	238	2.46	246	3.25
	231	3.19	239	2.52	247	2.55
	232	2.97	240	2.53	248	2.46
	233	3.12	241	2.39	249	2.14
	234	2.98	242	2.71	250	2.46
	235	3.69	243	2.71	251	2.64
	236	3.70	244	2.50	252	2.71
Average activity		3.41		2.53		2.64
" butter ingested, *gm.*		13.1		13.0		12.9
" cholesterol ingested, *mg.*			650		1290	

---

* Activity refers to per cent of the injected labeled phosphorus found as phospholipid. Each rat received 1 cc. of isotonic Na_2HPO_4.

† Rats weighed 160 to 185 gm. (females).

‡ Rats weighed 150 to 170 gm. (females).

§ Rats weighed 200 to 240 gm. (males).

two groups varied. One was fed 250 mg. of cholesterol in each 5 gm. of butter; the average amount of cholesterol ingested by this group over the 30 hour period was 545 mg. The other group was fed 500 mg. of cholesterol in each 5 gm. of butter; each rat of this group ingested an average of 963 mg. of cholesterol in the same period. The phospholipid activities of these two groups that had received cholesterol were respectively 2.50 and 2.31; the average control value was 3.01.

In Series 3, the procedure adopted was similar to that in Series 2. The animals used, however, were larger, and the total amount of cholesterol ingested during the experimental period of 30 hours was proportionately greater. The labeled phospholipid activities of the cholesterol-fed rats were 2.53 and 2.64, as compared with 3.41 for the control group.

Interrelation of Choline and Cholesterol in Their Effects upon Phospholipid Turnover of Liver—In view of the opposite effects of choline and cholesterol upon phospholipid metabolism, it seemed desirable to compare their actions in this respect when administered singly and together. The experiments recorded in Table II permit a comparison of the phospholipid turnover under the following three conditions: (1) cholesterol alone in rats fed a high fat diet, (2) choline alone in those fed a high fat diet, and (3) cholesterol and choline together in those fed a high fat diet.

The phospholipid activities of the four groups of rats shown in Table II were compared 4 hours after the P^{32} injection. The depressing effect of cholesterol upon the phospholipid turnover of the liver of the fat-fed rat is again brought out. Thus the average activities of the butter-fed rats and the cholesterol-fed rats were respectively 3.42 and 2.81. When animals maintained for 30 hours on the two diets received choline, the phospholipid activities of their livers were respectively 4.20 and 5.07. Choline increased the phospholipid activity of the liver of the butter-fed rats by 23 per cent, an increase in keeping with the values previously reported (1). In the cholesterol-fed rats, however, the same amount of choline was able to elevate the phospholipid activity of the liver from an initial value of 2.81 to 5.07, an increase of about 80 per cent. It is indeed striking that choline had a more pronounced effect in rats maintained on a cholesterol diet than in animals fed a butter diet, despite the fact that in the case of the cholesterol-fed animals choline was acting upon a phospholipid level already depressed below that observed in the animals fed the butter diet.

Duration of Action of Choline on Phospholipid Turnover in Liver of Rats Fed Cholesterol and Cholesterol-Free Diets—In a previous report it was shown that the stimulating effect of a single dose of choline chloride was of short duration, amounts of 25 mg. increasing phospholipid turnover for only about 10 to 15 hours in rats maintained on high fat diets (1). In the experiment described below, the time of the action of choline upon cholesterol-fed rats was determined and compared with that in butter-fed animals. 96 rats were divided into three groups and treated as follows:

TABLE II

Interrelation of Cholesterol and Choline upon Phospholipid Activity in Liver

The rats received the experimental diets (butter or cholesterol-butter) for 31 hours before removal of the liver for analyses. The rats weighed 150 to 180 gm. (females). Choline and P^{32} were administered simultaneously 4 hours before the livers were excised; *i.e.*, 27 hours after the rats were placed on the experimental diets. Choline was fed by stomach tube; the labeled phosphorus was injected subcutaneously. Each rat received 30 mg. of choline chloride in 1 cc. of solution and 1 cc. of isotonic Na_2HPO_4.

	Butter diet				Cholesterol-butter diet			
	Alone		With choline		Alone		With choline	
	Rat No.	Activity	Rat No.	Activity	Rat No.	Activity	Rat No.	Activity
	45	3.26	53	4.10	61	2.85	69	3.96
	46	3.31	54	4.05	62	2.46	70	4.85
	47	2.93	55	3.58	63	2.47	71	3.60
	48	2.90	56	4.36	64	3.16	72	5.42
	49	3.35	57	3.80	65	2.81	73	5.48
	50	3.63	58	4.83	66	2.18	74	5.63
	51	4.15	59	4.32	67	3.52	75	6.45
	52	3.86	60	4.54	68	3.00	76	5.16
Average activity		3.42		4.20		2.81		5.07
" butter ingested, *gm*		9.1		8.6		7.7		7.4
" cholesterol ingested, *mg*						453		440

1. Thirty-two rats were placed on a butter diet; at the end of 30 hours they received 30 mg. of choline chloride by stomach tube and radioactive phosphate subcutaneously. Groups of eight rats were killed at intervals of 4, 8, 16, and 30 hours thereafter. Access to the butter diet was permitted throughout the 60 hours of the experiment.

2. Thirty-two rats were fed the cholesterol diet for 30 hours and then received 30 mg. of choline chloride by stomach tube and radioactive phosphorus subcutaneously. They were killed at intervals of 4, 8, 16, and 30 hours thereafter, during which times they were permitted access to the experimental diet.

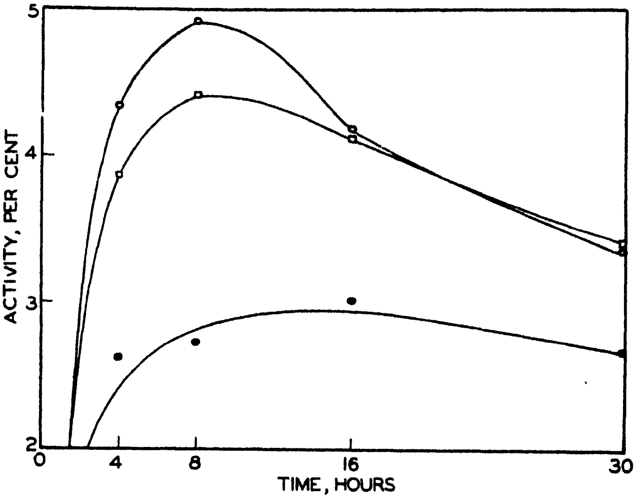


Fig. 1. The action of choline upon liver phospholipids of rats fed diets with and without cholesterol. The ordinates refer to per cent of administered labeled phosphorus found in the phospholipid of the whole liver. The abscissas refer to the time after phosphorus and choline administrations. ● represents values obtained from rats fed the cholesterol diet; ○ those from rats fed the cholesterol diet + choline; □ those from rats fed the butter diet + choline. Each point represents the average of eight separate liver analyses.

3. Thirty-two rats were fed the cholesterol diet for 30 hours before they were injected with phosphorus. *This group received no choline.* Livers were excised at 4, 8, 16, and 30 hour intervals.

The phospholipid activity as a function of time following choline and phosphorus administrations is shown in Fig. 1, which shows again that, when choline treatment is superimposed on a cholesterol diet, the stimulation in phospholipid activity is greater than when choline is added to a control diet containing butter alone. The duration of choline action appears to be the same in both cases.

DISCUSSION

The most striking finding brought out by the present investigation is the establishment of a close relation between cholesterol metabolism and phospholipid activity. The ingestion of cholesterol for only 30 hours depressed the liver content of the newly formed or labeled phospholipid, which was identified by a radioactive isotope of phosphorus. It is of considerable importance to note here that this depression in the content of labeled phospholipid occurred in livers at a time when they were still normal, for grossly abnormal lipid infiltrations do not appear in so short a period as 30 hours after the beginning of cholesterol feeding. The finding of a depressed labeled phospholipid content as early as 30 hours after the beginning of cholesterol feeding would seem to lend greater significance to this interrelation between cholesterol and phospholipid metabolism.

It was previously shown that choline speeds up phospholipid turnover in the livers of the rats fed high fat diets (1). Since the action of choline is also associated with the prevention of fatty livers, it seems reasonable to conclude that its action here is the result of an accelerated phospholipid turnover. On the other hand, the content of newly formed phospholipid in the liver is decreased by the feeding of cholesterol, which, if continued long enough, results in the production of fatty livers. Thus two processes, inverse in respect to their final effects, have been linked to the rate of phospholipid turnover: (1) the infiltration of abnormal amounts of fat observed under the influence of prolonged cholesterol feeding, an effect associated with a depressed phospholipid turnover in the liver; (2) the prevention of fatty livers by choline in rats fed high fat diets, an effect associated with an increased phospholipid turnover in the liver.

The influence of choline and cholesterol upon the total phospholipid content of the liver has been the subject of numerous investigations. The most extensive observations with respect to choline are those of Channon and his coworkers (9–12) who concluded that, in general, the total content of liver phospholipid is independent of dietary choline and the degree of fatty infiltration. Although both increases (13) and decreases (14) in total phospholipid of the liver have been noted after cholesterol feeding, the majority of workers (11, 15, 16) report no change when rats are subjected to this treatment. This constancy in *total phospholipid content* of the liver stands in marked contrast to the findings reported here, which show that the *phospholipid turnover* as measured by the level of newly formed phospholipid is accelerated and depressed by choline and cholesterol respectively. In the experiments reported here, measurements were concerned solely with newly formed phospholipid, synthesized during the short interval of 30 hours or less after the administration of P^{32}. This measurement differs from that of *total phospholipid,* which shows but little change in the liver even during times when enormous infiltrations of neutral fat are taking place. The remarkable tendency of the *total phospholipid content* of the liver to show but little change is particularly well demonstrated by the case of the completely depancreatized dog maintained with insulin, in which as much as 352 gm. of total lipid had accumulated in a liver weighing 770 gm. (a 35-fold increase in the whole liver above normal) without appreciably affecting the phospholipid content of this organ (7). This relative constancy in the *total phospholipid content of the liver* must not be taken as evidence of phospholipid inactivity. As already pointed out, the *total phospholipid content* may remain unchanged even while phospholipid activity, as judged by studies with radioactive phosphorus, is undergoing considerable change. The conclusion therefore seems warranted that the *total phospholipid content of the liver* is not necessarily an index of phospholipid activity or exchange.

The radioactive phosphorus used in this investigation was supplied by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due. The assistance furnished by the Works Progress Administration (Official Project No. 665-08-3-30, Unit A6) is also gratefully acknowledged.

SUMMARY

1. Cholesterol decreases phospholipid turnover in the liver. This effect was demonstrated in rats as early as 30 hours after the feeding of cholesterol was initiated.

2. Choline stimulates the phospholipid turnover in the liver of cholesterol-fed rats.

3. The acceleration in phospholipid activity produced by choline is more pronounced in the liver of the cholesterol-fed rat than in that of the fat-fed rat.

4. The relation of phospholipid turnover to fat deposition in the liver is discussed.

5. *Total phospholipid content* and *newly synthesized phospholipid* (labeled with radioactive phosphorus) are compared as indices of phospholipid activity in the liver.

BIBLIOGRAPHY

1. Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **127**, 211 (1939).
2. Schoenheimer, R., and Yuasa, D., *Z. physiol. Chem.*, **180**, 5 (1929).
3. Okey, R., *Proc. Soc. Exp. Biol. and Med.*, **30**, 1003 (1933).
4. Sperry, W. M., and Stoyanoff, V. A., *J. Nutrition*, **9**, 131 (1935).
5. Best, C. H., and Ridout, J. H., *J. Physiol.*, **78**, 415 (1933).
6. Best, C. H., Channon, H. J., and Ridout, J. H., *J. Physiol.*, **81**, 409 (1934).
7. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **108**, 201 (1935).
8. Perlman, I., Ruben, S., and Chaikoff, I. L., *J. Biol. Chem.*, **122**, 169 (1937-38).
9. Channon, J. H., Platt, A. P., Loach, J. V., and Smith, J. A. B., *Biochem. J.*, **31**, 2181 (1937).
10. Channon, J. H., Platt, A. P., and Smith, J. A. B., *Biochem. J.*, **31**, 1736 (1937).
11. Channon, J. H., and Wilkinson, H., *Biochem. J.*, **28**, 2026 (1934).
12. Beeston, A. W., Channon, H. J., and Wilkinson, H., *Biochem. J.*, **29**, 2659 (1935).
13. Stoesser, A. V., McQuarrie, I., and Anderson, J. A., *Proc. Soc. Exp. Biol. and Med.*, **33**, 595 (1936).
14. Loizides, P. A., *Biochem. J.*, **32**, 1345 (1938).
15. Okey, R., and Yokela, E., *J. Nutrition*, **11**, 463 (1936).
16. Aylward, F. X., and Stott, W., *Biochem. J.*, **31**, 2055 (1937).

THE CONCENTRATION OF IONIZED MAGNESIUM AND CALCIUM IN MILK

By R. NORDBÖ

(From the Department of Physiology, University of Oslo; Oslo, Norway)

(Received for publication, December 31, 1938)

A knowledge of the magnesium and calcium equilibria and of the concentration of ionized magnesium and calcium in milk is undoubtedly of importance to investigators of the physiology of milk secretion and of nutrition of infants. Our present knowledge of the physical chemistry of milk is in many respects far from satisfactory, and, as Clark (1935) states, "In the case of milk there are available not even the specific pK_a values for the several buffer systems, nothing on solubility products specifically applicable to milk, and few analytical data sufficient to the purpose."

In the first place an attempt was made to calculate the ionic strength of ultrafiltrate of milk. In ultrafiltrates of fresh market milk there were determined the concentrations of Na, K, Ca, Mg, inorganic phosphate, Cl, and citric acid.

Sodium was determined by the method of Barrenscheen and Messiner (1927) with the following modifications: (1) Powdered $Ca(OH)_2$ was employed to precipitate phosphate, as recommended by Butler and Tuthill (1931), and (2) the precipitated uranyl sodium zinc acetate was washed with 96 per cent alcohol saturated with the triple salt at room temperature, as recommended by Barber and Kolthoff (1928). According to Barber and Kolthoff, about 0.5 mg. of the triple salt is dissolved in 1 cc. of a saturated solution in 95 per cent alcohol at 22°.

Procedure

Place the ultrafiltrate and 1 drop of a 1 per cent alcoholic solution of phenolphthalein plus $Ca(OH)_2$ in a small, stoppered flask. Shake and let stand 30 minutes with occasional shaking. Filter on an ashless filter paper (*e.g.*, Schleicher and Schüll, Blue Ribbon). Into a Jena glass filter tube of the form previously described (Nordbö, 1939) are pipetted 1.5 cc. of freshly filtered uranium zinc acetate reagent and 0.5 cc. of the phosphate-free ultrafiltrate. Then 0.5 cc. of 96 per cent alcohol is added drop by drop, with shaking after each drop. Let stand 30 minutes. The mother liquor is completely removed through the glass filter, but suction is not applied so long that air is drawn through the filter. The precipitate is then washed once with 0.5 cc. and three times with 1 cc. of the 96 per cent alcohol saturated with the triple salt and filtered before use. Then 1 cc. of water and 1 cc. of approximately 5 per cent acetic acid are sucked through the glass filter into the tube. More water is added directly, and the contents are mixed until the precipitate is completely dissolved. Transfer to a 50 cc. volumetric flask and dilute with water to the mark.

Standard—In another 50 cc. flask are measured 5 cc. of a uranyl sodium zinc acetate solution in 1 per cent acetic acid containing about 270 mg. of the salt in 100 cc. Water is added to the mark. Then 0.5 cc. of a 20 per cent potassium ferrocyanide solution is added to standard and unknown, the contents are mixed, and the solutions compared colorimetrically.

Calculation—66.9 mg. of the triple salt = 1 mg. of Na. 0.5 cc. samples of a salt solution containing 1.000 gm. of NaCl, 1.5 gm. of KH_2PO_4, and 2 gm. of citric acid per liter were analyzed with the following results: 199, 201, and 197 micrograms of Na (theory, 197 micrograms of Na).

In ultrafiltrate of three samples of the market milk in Oslo the following values were found: 20.6, 20.0, and 19.3 mM of Na per liter.

Potassium was precipitated as $K_2NaCo(NO_2)_6$ by the separate addition of $NaNO_2$ and $Co(NO_3)_2$, as recommended by Bonneau (1929) and by Jendrassik and Szél (1933). The precipitation was carried out in Jena glass filter tubes of the form used for sodium and the precipitate dissolved and titrated according to the procedure recommended by Leulier *et al.* (1928). In the ultrafiltrate of three samples of the market milk in Oslo the following values were found: 41.5, 41.3, and 40.8 mM of K per liter. *Total calcium* was determined by permanganate titration of calcium oxalate; *total magnesium* by Berg's oxin method (Nordbö, 1939);

inorganic phosphate by the method of Fiske and Subbarow (1925); *pH*, colorimetrically; *chloride* by the method of Van Slyke (1923-24). The concentration of Cl in ultrafiltrate of the market milk in Oslo was found to be very constant, between 31 and 32 mM per liter. *Total citrate* was determined as pentabromo acetone according to the procedure recommended by Reichard (1934). The precipitate was dissolved and titrated by the method of Kometiani (1931). Precipitation and titration were carried out in 30 cc. glass filter tubes, of the same form as the smaller tubes used for Na, K, Mg, and Ca. In this way it was possible to determine as little as 1 to 2 mg. of citric acid with an error within ±3 per cent.

As a first approximation in the calculation of the ionic strength the following assumptions were made.

(*a*) Calcium and magnesium were supposed to react with trivalent citrate ions ($\bar{C}'''$)[1] according to the equations first proposed by Hastings *et al.* (1934).

$$\frac{[Ca^{\cdot\cdot}][\bar{C}''']}{[Ca\bar{C}']} = 6 \times 10^{-4} \tag{1}$$

$$\frac{[Mg^{\cdot\cdot}][\bar{C}''']}{[Mg\bar{C}']} = 6 \times 10^{-4} \tag{2}$$

The numerical value, 6×10^{-4}, originally calculated for $t = 22°$ and $\mu = 0.15$, was taken to be valid in ultrafiltrate of milk at 37°.

(*b*) Only a negligible part of the calcium citrate complex and of the magnesium citrate complex was considered to be present as undissociated $Ca\bar{C}H$ and $Mg\bar{C}H$ at the pH of ultrafiltrate of milk, about 6.8, and the greater part to be present as the ions $Ca\bar{C}'$ and $Mg\bar{C}'$.

(*c*) It was assumed that there are no other non-ionized magnesium and calcium compounds in ultrafiltrate of milk than the citrate compounds mentioned above. Therefore

$$[Ca^{\cdot\cdot}] + [Ca\bar{C}'] = \text{total calcium} \tag{3}$$

$$[Mg^{\cdot\cdot}] + [Mg\bar{C}'] = \text{total magnesium} \tag{4}$$

---

[1] Throughout the paper the symbol $\bar{C}$ will be used to represent citrate.

(*d*) All possible forms of citric acid other than $\text{Mg}\bar{\text{C}}'$, $\text{Ca}\bar{\text{C}}'$ and $\bar{\text{C}}'''$ were disregarded; *e.g.*, $\text{Mg}\bar{\text{C}}\text{H}$, $\text{Ca}\bar{\text{C}}\text{H}$, and $\text{H}\bar{\text{C}}''$. Therefore

$$(5) \qquad [\text{Mg}\bar{\text{C}}'] + [\text{Ca}\bar{\text{C}}'] + [\bar{\text{C}}'''] = \text{total citric acid}$$

(*e*) Since the value of pK'_2 for phosphoric acid in ultrafiltrate of milk has never been determined, the value of pK'_2 for Sørensen's M/15 phosphate solution at pH 6.81 and 20° was chosen; *i.e.*, $\text{pK}'_2 = 6.81$.

By means of Equations 1 to 5 and the values found for total calcium (8.7 mM per liter), total magnesium (2.6 mM per liter), and total citrate (9.5 mM per liter) the following values were calculated: $[\text{Mg}^{\cdot\cdot}] = 0.75$, $[\text{Mg}\bar{\text{C}}'] = 1.85$, $[\text{Ca}^{\cdot\cdot}] = 2.5$, $[\text{Ca}\bar{\text{C}}'] = 6.2$, and $[\bar{\text{C}}'''] = 1.48$ mM per liter of ultrafiltrate.

Based on the determined and calculated concentrations of the different ionic species, the ionic strength (μ) of the milk ultrafiltrate was estimated to be 0.078. The contribution of the polyvalent ions to the total ionic strength was calculated to be 0.024.

Dissociation Constant of Magnesium Citrate and Calcium Citrate at 37° and μ 0.08

The solubility of the calcium and of the magnesium compound of tropeolin OO (CaTrop_2 and MgTrop_2) was determined in salt solutions of known concentrations of calcium ions and magnesium ions, respectively, and at 37°. The desired concentrations of calcium ions and magnesium ions, 0.3 to 2.5 mM per liter, were obtained by the addition of CaCl_2 and MgCl_2. These salts were considered to be completely ionized. The ionic strength of the solutions examined was adjusted to 0.08 by the addition of NaCl and K_2SO_4. So much of K_2SO_4 was added that the ionic strength of the polyvalent ions was about 0.024.

The solutions were shaken with an excess of CaTrop_2 or of MgTrop_2 in 20 cc. Jena flasks equipped with special stoppers.[2] The flasks were rotated for 3 hours in a water bath at 37° (±0.1°). The flasks were then taken out of the water bath, wiped off on the outside, and the contents filtered through a glass filter, as previously described (Nordbö, 1938). The filtration of the solutions saturated with $CaTrop_2$ was carried out in an air thermostat at 37° and 1 cc. of the filtrates was diluted to 10 cc. with distilled water. These dilutions may be dealt with at room temperature without any risk of precipitation of $CaTrop_2$ being incurred. The concentration of the dye in the filtered, saturated solutions of $MgTrop_2$ and in the diluted solutions of $CaTrop_2$ was determined in the Duboscq colorimeter by comparison with a standard solution of the potassium salt of tropeolin OO (Nordbö, 1939). From the results of the solubility experiments carried out (thirty-four experiments) the following ***solubility products*** were calculated.

---

[2] Jena Glassworks, catalogue No. 4176-b.

$$[Mg^{\cdot\cdot}]\,[Trop']^2 = 1.60 \times 10^{-13} \tag{6}$$

$$[Ca^{\cdot\cdot}]\,[Trop']^2 = 2.42 \times 10^{-11} \tag{7}$$

In some preliminary experiments the value of Equations 1 and 2 was found to be about 3×10^{-4} at 37° and μ 0.08. The final experiments were made with salt solutions of a total ionic strength of 0.08 and a concentration of trivalent citrate ions of about 1.5 mM per liter; *i.e.*, the concentration expected in ultrafiltrate of milk. By adding K_2SO_4 the contribution of the polyvalent ions to the ionic strength was adjusted to about 0.024. Total citrate in the solutions was 3 to 11.5 mM per liter and total calcium or magnesium 1.8 to 9.9 mM per liter. The pH in the solutions was kept at about 7.5. The solutions containing calcium were saturated with $CaTrop_2$ and the solutions containing magnesium with $MgTrop_2$ as described above. From the estimated concentrations of the dye in the saturated solutions the concentration of magnesium and calcium ions was calculated by means of Equations 6 and 7 respectively.

In the experiments with magnesium the concentrations of $Mg\bar{C}'$ and $\bar{C}'''$ are given by Equations 8 and 9.

$$[Mg\bar{C}'] = [MgCl_2] + [MgTrop_2] - [Mg^{\cdot\cdot}] - [Mg\bar{C}H] \tag{8}$$

$$[\bar{C}'''] = [Na_3\bar{C}] - [Mg\bar{C}'] - [Mg\bar{C}H] - [H\bar{C}''] \tag{9}$$

The corresponding equations for the calcium experiments are

$$[Ca\bar{C}'] = [CaCl_2] + [CaTrop_2] - [Ca^{\cdot\cdot}] - [Ca\bar{C}H] \tag{10}$$

$$[\bar{C}'''] = [Na_3\bar{C}] - [Ca\bar{C}'] - [Ca\bar{C}H] - [H\bar{C}''] \tag{11}$$

$[MgCl_2]$, $[CaCl_2]$, and $[Na_3\bar{C}]$ represent the concentrations of $MgCl_2$, $CaCl_2$, and $Na_3\bar{C}$ added, $[MgTrop_2]$ and $[CaTrop_2]$ the concentrations of $\mathrm{MgTrop_2}$ and $\mathrm{CaTrop_2}$ dissolved. At pH about 7.5 the corrections due to the $[\mathrm{Mg\bar{C}H}]$, $[\mathrm{Ca\bar{C}H}]$, and $[\mathrm{H\bar{C}''}]$ terms are certainly so small that they may be neglected in Equations 8 to 11.

The arithmetical mean of thirty-seven values found for the expression $[\mathrm{Mg}^{\cdot\cdot}]\,[\mathrm{\bar{C}'''}]/[\mathrm{Mg\bar{C}'}]$ was 2.65×10^{-4} and of twenty-four values found for the expression $[\mathrm{Ca}^{\cdot\cdot}]\,[\mathrm{\bar{C}'''}]/[\mathrm{Ca\bar{C}'}]$ was 3.16×10^{-4}. This means that $\mathrm{Mg\bar{C}'}$ and $\mathrm{Ca\bar{C}'}$ are less dissociated at 37° and μ 0.08 than at 22° and μ 0.15 (where the dissociation constant is about 6×10^{-4}). This is, indeed, what might be expected, since the *activity* of the trivalent citrate ion is much more influenced by a change in the ionic strength than is the activity of the monovalent $\mathrm{Mg\bar{C}'}$ or $\mathrm{Ca\bar{C}'}$.

Combination of Lactose with Calcium and Magnesium

Herrington (1934) isolated from concentrated solutions of lactose and $\mathrm{CaCl_2}$ a compound of the composition α-lactose · $\mathrm{CaCl_2 \cdot 7H_2O}$. He also found an increased solubility of lactose in concentrated $\mathrm{CaCl_2}$ solutions and a shift in specific rotation dependent "upon the concentration of both salt and sugar in the way which would be expected if a compound did exist in solution."

In order to find out to what extent such a combination of lactose and Ca or Mg exists in salt solutions of a composition similar to that of ultrafiltrate of milk, determination of $[\mathrm{Ca}^{\cdot\cdot}]$ or $[\mathrm{Mg}^{\cdot\cdot}]$ was made in salt solutions containing 0.15 mole of lactose and 2 to 4 mM of $\mathrm{CaCl_2}$ or $\mathrm{MgCl_2}$ per liter and NaCl to give a supposed ionic strength of about 0.08. The solubility of $\mathrm{CaTrop_2}$ and $\mathrm{MgTrop_2}$ in these solutions was determined and the concentration of $\mathrm{Mg}^{\cdot\cdot}$ and of $\mathrm{Ca}^{\cdot\cdot}$ calculated from Equations 6 and 7 respectively. The concentration of the magnesium-lactose compound, [lactose · Mg], and of the calcium-lactose compound, [lactose · Ca], is given by Equations 12 and 13.

$$[\mathrm{Lactose \cdot Mg}] = [\mathrm{MgCl_2}] - [\mathrm{Mg}^{\cdot\cdot}] + [\mathrm{MgTrop_2}] \tag{12}$$

$$[\mathrm{Lactose \cdot Ca}] = [\mathrm{CaCl_2}] - [\mathrm{Ca}^{\cdot\cdot}] + [\mathrm{CaTrop_2}] \tag{13}$$

$[\mathrm{MgCl_2}]$ and $[\mathrm{CaCl_2}]$ represent the concentration of $\mathrm{MgCl_2}$ and $\mathrm{CaCl_2}$ added, $[\mathrm{MgTrop_2}]$ and $[\mathrm{CaTrop_2}]$ the concentration of $\mathrm{MgTrop_2}$ and $\mathrm{CaTrop_2}$ dissolved. If it be assumed that 1 molecule of lactose binds 1 molecule of Ca or 1 molecule of Mg, then in the experiments carried out the concentration of lactose·Ca or of lactose·Mg represents less than 1 per cent of the total concentration of lactose. The concentration of lactose that is not combined with Ca or Mg, [lactose], is then practically equal to the total concentration of lactose; *i.e.*, 0.15 mole per liter. This concentration of lactose represents the average concentration in skim milk (Wright, 1935).

It was found by the experiments that lactose combines with Ca and Mg to form soluble, non-ionized compounds. The concentration of the non-ionized compounds depends upon the concentration of both lactose and $Ca^{\cdot\cdot}$ or $Mg^{\cdot\cdot}$. This may be expressed by Equations 14 and 15.

$$(14) \qquad q_1 \quad \frac{[Ca^{\cdot\cdot}]\,[\text{lactose}]}{[\text{lactose}\cdot Ca]}$$

$$(15) \qquad q_2 \quad \frac{[Mg^{\cdot\cdot}]\,[\text{lactose}]}{[\text{lactose}\cdot Mg]}$$

The value of q averages 0.3 (at 37° and μ 0.08) and gives the impression of being a constant. The deviations from the mean are within the limits of experimental error, an error of +10 per cent in the *determination* of $[Ca^{\cdot\cdot}]$ and $[Mg^{\cdot\cdot}]$, giving an error of −20 per cent in the *calculation* of [lactose·Ca] and [lactose·Mg]. It is possible that lactose·Ca and lactose·Mg are in equilibrium with a special form of lactose which represents only a part of the total lactose in the solution. Further experiments are needed to show whether or not the concentration of lactose·Ca and lactose·Mg is influenced by change of temperature, pH, and ionic strength.

State of Calcium and Magnesium in Ultrafiltrate of Fresh Cow's Milk

The ultrafiltrates were prepared at about 3° by means of Wilenski (1929) glass filter candles coated with collodion. The volume of ultrafiltrate was less than 5 per cent of the volume of milk from which it was prepared. The process of ultrafiltration therefore caused only a negligible change in the composition of ultrafiltrate and milk. The results of the analyses are given in Table I. Ionized magnesium was determined by saturation with $MgTrop_2$ as described earlier in this paper. Lactose was not determined in the same ultrafiltrates, for the reason that it was the result of the analyses that made it probable that lactose combines with calcium and magnesium. It was found upon analysis of separate samples of the market milk in Oslo that the concentration of lactose in the ultrafiltrate amounted to 5.25 gm. per 100 cc.; *i.e.*, 0.146 mole per liter. The method of Jacobsen (1938) was used. In calculating the concentration of lactose·Mg and lactose·Ca (Table II), the concentration of lactose was assumed to be 0.15 mole per liter in all the ultrafiltrates. The results of the calculations of the concentration of the different fractions of calcium, magnesium, and citric acid in the ultrafiltrates in Table I are given in Table II.

TABLE I

Analysis of Ultrafiltrate of Milk

The results are expressed in mM per liter.

Milk No.	Ultra-filtrate No.	Total Mg	$[Mg^{\cdot\cdot}]$	Total Ca	Total citrate	Inorganic phosphate	pH
I	F_2	2.53	0.46	8.54	8.94	11.35	6.77
	F_3	2.48	0.48	8.63	9.12		6.77
					9.20		
	F_4	2.49	0.48	8.68	8.83		6.75
		2.54	0.49		9.37		
	F_5		0.45	8.68	9.28		6.75
			0.44		9.40		
	F_6				9.08	11.90	
					9.54		
	F_7					11.85	
II	F_2	2.65	0.47	9.70	9.13		6.73
		2.67	0.47	9.70	8.94		
	F_3					11.40	6.70
						11.40	

Calculation of Ionic Strength of Ultrafiltrate of Milk

The results of the calculation of the ionic strength of Ultrafiltrate I-F_2 are given in Table III. The concentration of inorganic phosphate was 11.35 mM per liter and $[H^{\cdot}]$ was 1.70×10^{-7}. The second dissociation constant of phosphoric acid was calculated from the formula $pK'_2 = 7.15 - 1.25 \sqrt{\mu}$ (Sendroy and Hastings, 1926–27). Assuming the ionic strength of the ultrafiltrate to

TABLE II

Magnesium and Calcium Distribution in Ultrafiltrate of Milk

The results are expressed in mM per liter.

Milk No.	Ultrafiltrate No.	$[Mg^{\cdot\cdot}]$	[Lactose·Mg]	$[Mg\bar{C}']$	$[Mg\bar{C}H]$	$[\bar{C}''']$	$[H\bar{C}'']$	$[Ca\bar{C}']$	$[Ca\bar{C}H]$	$[Ca^{\cdot\cdot}]$	[Lactose·Ca]	Ca balance
I	F_2	0.46	0.23	1.82	0.02	1.05	0.13	5.87	0.05	1.77	0.83	−0.03
	F_3	0.48	0.24	1.74	0.02	0.96	0.12	6.27	0.05	2.06	1.03	−0.78
	F_4	0.48	0.24	1.77	0.02	0.98	0.13	6.15	0.05	1.98	0.99	−0.49
II	F_2	0.47	0.23	1.94	0.02	1.10	0.15	5.78	0.05	1.66	0.83	+1.38

As an illustration the calculations for Ultrafiltrate I-F_2 are given here.

$$[\text{Lactose}\cdot\text{Mg}] = \frac{[Mg^{\cdot\cdot}]\,[\text{lactose}]}{0.3} = \frac{0.46 \times 10^{-3} \times 0.15}{0.3}$$

$$= 0.23 \times 10^{-3} \text{ mole per liter}$$

$$[Mg\bar{C}'] + [Mg\bar{C}H] = [\text{total Mg}] - [Mg^{\cdot\cdot}] - [\text{lactose}\cdot\text{Mg}]$$

$$= (2.53 - 0.43 - 0.23) \times 10^{-3} = 1.84 \times 10^{-3} \text{ mole per liter}$$

$$\frac{[Mg\bar{C}H]}{[Mg\bar{C}']} = \frac{[H^{\cdot}]}{2 \times 10^{-5}} = \frac{1.7 \times 10^{-7}}{2 \times 10^{-5}} \text{ whence } [Mg\bar{C}H] = \text{about } 0.02 \times 10^{-3} \text{ mole}$$

$$\text{per liter and } [Mg\bar{C}'] = 1.82 \times 10^{-3} \text{ mole per liter}$$

$$[\bar{C}'''] = \frac{[Mg\bar{C}']}{[Mg^{\cdot\cdot}]} \times 2.65 \times 10^{-4} = \frac{1.82 \times 10^{-3}}{0.46 \times 10^{-3}} \times 2.65 \times 10^{-4}$$

$$1.05 \times 10^{-3} \text{ mole per liter}$$

$$[HC''] = \frac{[H^{\cdot}]\,[\bar{C}''']}{1.35 \times 10^{-6}} = \frac{1.7 \times 10^{-7} \times 1.05 \times 10^{-3}}{1.35 \times 10^{-6}} = 0.13 \times 10^{-3} \text{ mole per liter}$$

$$[Ca\bar{C}'] + [Ca\bar{C}H] = [\text{total citric acid}] - [Mg\bar{C}'] - [Mg\bar{C}H] - [\bar{C}'''] - [H\bar{C}'']$$

$$= (8.94 - 1.82 - 0.02 - 1.05 - 0.13)$$

$$\times 10^{-3} = 5.92 \times 10^{-3} \text{ mole per liter}$$

$$\frac{[Ca\bar{C}H]}{[Ca\bar{C}']} = \frac{[H^{\cdot}]}{2 \times 10^{-5}} = \frac{1.7 \times 10^{-7}}{2 \times 10^{-5}} \text{ whence } [Ca\bar{C}H] = \text{about } 0.05 \times 10^{-3} \text{ mole}$$

$$\text{per liter and } [Ca\bar{C}'] = 5.87 \times 10^{-3} \text{ mole per liter}$$

TABLE II—*Concluded*

$$[\mathrm{Ca}^{\cdot\cdot}] = \frac{[\mathrm{Ca}\bar{\mathrm{C}}']}{[\bar{\mathrm{C}}''']} \times 3.16 \times 10^{-4} = \frac{5.87 \times 10^{-3}}{1.05 \times 10^{-3}} \times 3.16 \times 10^{-4}$$

$$= 1.77 \times 10^{-3} \text{ mole per liter}$$

$$[\mathrm{Lactose}\cdot\mathrm{Ca}] \quad \frac{[\mathrm{Ca}^{\cdot\cdot}]\,[\mathrm{lactose}]}{0.3} = \frac{1.77 \times 10^{-3} \times 0.15}{0.3}$$

$$= 0.88 \times 10^{-3} \text{ mole per liter}$$

$$\text{Calcium balance} = [\text{total Ca}] - [\text{lactose}\cdot\text{Ca}] - [\mathrm{Ca}\bar{\mathrm{C}}'] - [\mathrm{Ca}\bar{\mathrm{C}}\mathrm{H}] - [\mathrm{Ca}^{\cdot\cdot}]$$

$$= (8.54 - 0.88 - 5.87 - 0.05 - 1.77)$$

$$\times 10^{-3} = -0.03 \times 10^{-3} \text{ mole per liter}$$

TABLE III

Corrected Ionic Strength of Ultrafiltrate of Milk

	Concentration	Ionic strength	Anions	Cations
	mM per l.	*mM per l.*	*m.eq. per l.*	*m.eq. per l.*
$\mathrm{Na}^{\cdot}$	20	10.0		20.00
$\mathrm{K}^{\cdot}$	42	20.5		42.00
$\mathrm{Ca}\bar{\mathrm{C}}'$	5.87	2.9	5.87	
$\mathrm{Mg}\bar{\mathrm{C}}'$	1.82	0.9	1.82	
$\mathrm{H_2PO_4}'$	5.88	2.9	5.88	
Cl'	32	16.0	32.00	
$\mathrm{Mg}^{\cdot\cdot}$	0.46	0.9		0.92
$\mathrm{Ca}^{\cdot\cdot}$	1.77	3.5		3.54
$\mathrm{HPO_4}''$	5.47	11.0	10.94	
$\bar{\mathrm{C}}'''$	1.05	4.7	3.15	
Undetermined anions			6.80	
Sum		73.3	66.46	66.46

be about 0.08, $\mathrm{p}K'_2 = 7.15 - 1.25\sqrt{0.08} = 6.80$ and $K'_2 = 1.58 \times 10^{-7}$. Then

$$\frac{[\mathrm{HPO_4}'']}{[\mathrm{H_2PO_4}']} = \frac{1.58 \times 10^{-7}}{1.70 \times 10^{-7}} = \frac{5.47 \times 10^{-3}}{5.88 \times 10^{-3}}$$

If we assume that all of the sodium, potassium, and chloride is in the form of ions, and that the inorganic phosphate, as determined by the method of Fiske and Subbarow, is present only as $\mathrm{H_2PO_4}'$ and $\mathrm{HPO_4}''$, then the ionic strength of the ions of which the concentration is known from analysis and calculation is estimated to be 0.0733. From the last two columns of Table III it is seen that the sum of the bases exceeds the sum of the acid equivalents. The difference, 6.8 milliequivalents per liter, is supposed to be represented by SO_4'', HCO_3', lactate′, etc. Assuming the ionic strength of these ions to be about 0.005, the total ionic strength of Ultrafiltrate I-F_2 is about 0.078. The value previously chosen for the ionic strength of ultrafiltrate of milk (μ 0.08) must therefore be regarded as being approximately correct and the calculations made on the assumption that $\mu = 0.08$ need not be repeated. Whether the ionic strength of whole milk (calculated per kilo of water) approximates that of the ultrafiltrate depends upon the manner in which it is influenced by the electric charge of the non-diffusible ions (Van Slyke *et al.*, 1925).

Comment

It is of interest to note that the concentration of ionized magnesium found in ultrafiltrate of market milk, 0.4 to 0.5 mm per liter, is the same as previously found in ultrafiltrate of blood serum (Nordbö, 1939), whereas total magnesium was found to be about 2.5 and 0.7 mm per liter respectively. Furthermore, the values calculated for the concentration of ionized calcium in ultrafiltrate of milk approximate the values for total calcium in ultrafiltrate of blood serum. Practically all of the calcium in ultrafiltrate of blood serum is present in the form of calcium ions (McLean and Hastings, 1934). In ultrafiltrate of milk the concentration of total calcium was found equal to 8.5 to 10 mm per liter; *i.e.*, the ionized calcium amounts to about 20 per cent of the total calcium. The concentration of citric acid and of lactose is of the order of magnitude necessary to account for the non-ionized magnesium and calcium, assuming the concentration of ionized magnesium and calcium to be equal to that in ultrafiltrate of blood serum. This fact is undoubtedly of importance when the complicated question of milk secretion is being discussed. It would be desirable to make simultaneous analyses of milk and blood serum from the same individual. It is also considered important to carry out analyses of milk samples obtained in successive stages of milking in order to see whether there are any fixed relations among the concentrations of citric acid, lactose, ionized and non-ionized magnesium and calcium. In considering the question of the nutritive properties of milk as food for suckling infants it is of importance to bear in mind that both lactose and citric acid combine with calcium and magnesium to form non-ionized compounds, thereby reducing the risk of phosphate precipitation in the intestine. Shohl (1937) found that by feeding rats with a citric acid-sodium citrate mixture in addition to otherwise rachitogenic diets the development of rickets was prevented. Kline *et al.* (1932) and French and Cowgill (1937) found that the presence of lactose in the diet favorably influences the utilization of calcium and phosphorus by the *growing* organism. Siwe (1938) suggests the use of undiluted cow's milk to which citric acid has been added as a substitute diet for infants. Meyer (1927) and Weissenberg (1928) also recommend a "citric acid milk." It is known that the addition of citric acid to cow's milk produces a more finely divided casein precipitate. If this is due to a reduction in the concentration of calcium ions, a similar effect might be expected from the addition of extra lactose.

SUMMARY

1. The results of analyses of K, Na, Ca, Mg, Cl, inorganic phosphate, and citric acid and of the determination of [H˙] and [Mg˙˙] indicate a value for the ionic strength of about 0.075 to 0.08 in ultrafiltrate of fresh market milk.

2. At 37° and μ 0.08 the dissociation constant of calcium citrate was found to be 3.16×10^{-4} and of magnesium citrate 2.65×10^{-4}.

3. It has been shown that lactose combines with calcium and magnesium to form non-ionized compounds.

4. The concentration of magnesium ions in ultrafiltrates of fresh market milk is 0.4 to 0.5 mM per liter. Total diffusible magnesium is about 2.5 mM per liter.

5. Of the diffusible calcium of fresh market milk (8.5 to 10 mM per liter) only about 20 per cent is present as calcium ions.

6. The importance of citric acid and of lactose for the absorption of phosphate, calcium, and magnesium by the growing organism has been discussed.

BIBLIOGRAPHY

Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, **50**, 1625 (1928)
Barrenscheen, H. K., and Messiner, L., *Biochem. Z.*, **189**, 308 (1927).
Bonneau, L., *Bull. Soc. chim. France*, **45**, 798 (1929).
Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, **93**, 171 (1931).

Clark, W. M., in Rogers, L. A., chap. 6, 145.

Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).

French, R. B., and Cowgill, G. R., *J. Nutrition*, **14**, 383 (1937).

Hastings, A. B., McLean, F. C., Eichelberger, L., Hall, J. L., and Da Costa, E., *J. Biol. Chem.*, **107**, 351 (1934).

Herrington, B. L., *J. Dairy Sc.*, **17**, 701, 805 (1934).

Jacobsen, A. P., *Skand. Arch. Physiol.*, **79**, 87 (1938).

Jendrassik, L., and Szél, I., *Biochem. Z.*, **267**, 124 (1933).

Kline, O. L., Keenan, J. A., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **98**, 121 (1932).

Kometiani, P. A., *Z. anal. Chem.*, **86**, 359 (1931).

Leulier, A., Velluz, L., and Griffon, H., *Bull. Soc. chim. biol.*, **10**, 891 (1928).

McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, **108**, 285 (1934).

Meyer, S., *Arch. Kinderheilk.*, **82**, 241 (1927).

Nordbö, R., *Skand. Arch. Physiol.*, **80**, 341 (1938); **81**, 258, 263, 265 (1939).

Reichard, O., *Z. anal. Chem.*, **99**, 81 (1934).

Rogers, L. A., associates of, Fundamentals of dairy science, New York, 2nd edition (1935).

Sendroy, J., Jr., and Hastings, A. B., *J. Biol. Chem.*, **71**, 783 (1926–27).

Shohl, A. T., *J. Nutrition*, **14**, 69 (1937).

Siwe, S., *Skand. Arch. Physiol.*, **80**, 385 (1938).

Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923-24).

Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **65**, 701 (1925).

Weissenberg, H., *Arch. Kinderheilk.*, **84**, 200 (1928).

Wilenski, B. A., *Biochem. Z.*, **204**, 433 (1929).

Wright, P. A., in Rogers, L. A., chap. 1, 35.

THE ABSORPTION SPECTRA OF THE COMPOUNDS FORMED BY ANDROSTERONE AND TESTOSTERONE IN THE *m*-DINITROBENZENE REACTION

By G. O. LANGSTROTH and N. B. TALBOT

(From the George Eastman Laboratories of Physics, Massachusetts Institute of Technology, and from the Biological Laboratories, Harvard University, Cambridge, and the Department of Pediatrics, Harvard Medical School, Boston)

(Received for publication, March 4, 1939)

There is need for more satisfactory methods for the quantitative and qualitative determination of the steroid sex hormones. It appears that these methods must be of a chemical nature, for although biological methods may always have the advantage in certainty in proving the presence of physiologically active material, they show little promise of qualitative accuracy. Qualitative methods of assay are required because of the marked differences in biological activity of some of the hormones (1, 2).

Several attempts have been made to develop methods of androgen assay based on the colorimetric estimation of the compounds formed when solutions containing androgens are treated with *m*-dinitrobenzene in the presence of an alkali (3–9). The evidence thus far presented shows that the correlation between the colorimetric and biological assays is not as good as one could wish. The obvious difficulties are that (*a*) the colorimetric measurements include the effects produced in the chosen wave-length region by all urinary compounds present, and (*b*) no distinction is made between the various hormones which may differ markedly in biological activity. It is apparent that, until some method of distinguishing between the various hormones is devised, marked discrepancies may be expected to appear in a correlation of chemical with biological assays.

In an attempt to obtain information which might be useful in development of methods of androgen assay, a study has been made of the absorption spectra of the compounds formed in the *m*-dinitrobenzene reaction in alcoholic solutions of synthetic androsterone and testosterone. These hormones were chosen for the initial study because they differ greatly in biological activity; consequently it is important to be able to distinguish between them. Knowledge concerning such spectra is extremely limited; some rough curves obtained with filters have been published (4, 8), but these extend only over the wave-length region λ 4300 to 6100. The results of the preliminary investigation reported in this article cover the range λ 2800 to 9000, and indicate that such studies may be important in development of satisfactory methods of assay. They show that reasonably accurate determinations of the amounts of synthetic androsterone and testosterone in an alcoholic solution can be obtained readily from examination of the absorption spectrum of the reaction mixture. While difficulties are to be expected in the application of the results to the analysis of urine extracts, the findings provide a promising basis on which to work.

EXPERIMENTAL

m-Dinitrobenzene Reaction—The reaction conditions employed in this work were very similar to those used by Callow *et al.* (8). A measured quantity of a solution of androsterone, or of testosterone, or both, in absolute alcohol was placed in a test-tube, and the volume brought up to 0.20 cc. by addition of alcohol. 0.20 cc. of a solution of *m*-dinitrobenzene, and then 0.20 cc. of a 2.5 N KOH solution, both in absolute alcohol, were added. The mixture, together with a blank prepared in a similar way but containing no hormone, was placed in a constant temperature bath at 25.1° ± 0.1°, and allowed to develop for the desired length of time. On removal from the bath the test and blank reaction mixtures were diluted to an appropriate volume with absolute alcohol, and transferred to absorption cells for spectroscopic examination. This was completed within 15 minutes after dilution of the mixtures; no change in the absorption characteristics was observed within this time. For the major part of the investigation the concentration of *m*-dinitrobenzene in the stock solution was 11.6 mg. per cc., and the development time was 80 minutes; the dilution at the end of the development was usually made by adding 4.0 cc. of absolute alcohol.

Reagents—Highest grade commercial absolute alcohol was used throughout the investigation. High grade *m*-dinitrobenzene (Eastman Kodak Company) and KOH (Merck's reagent) were used; the former was further purified, and the latter was made up and checked for correct concentration, according to the directions given by Callow *et al.* (8). Fresh stock solutions of both were made up each week, and when not in use were kept in the dark at low temperature. They showed no trace of discoloration during the period in which they were used.

Hormones—Samples of crystalline androsterone and testosterone were obtained through the kindness of Dr. Erwin Schwenck of the Schering Corporation. They were made up in known solutions with absolute alcohol, without further purification.

Determination of Absorption Curves—The extinction-wave-length curves for the test mixtures minus those for the corresponding blanks were determined photographically with the aid of a Spekker photometer and a Hilger E-2 quartz spectrograph. The light source for observations up to λ 7000 consisted of a condensed spark between tungsten-steel or vanadium-iron electrodes, while for observations at greater wave-lengths an incandescent source was employed. Eastman Kodak No. 1-F and No. 1-L photographic plates were used. The quartz absorption cells contained a layer of solution 1 cm. thick.

A series of spectra, in which the intensity of the light beam passing through the blank solution was varied in steps corresponding to an extinction difference of 0.1 at important points, was taken for each reaction mixture; by interpolation between these spectra extinction values could be read off to 0.03.

It was found that a change (from 4 to 10 cc.) in the amount of alcohol added to a reaction mixture at the end of the development period produced no detectable effect on the shape of the extinction curve; and that the extinction values agreed with those expected from a consideration of Beer's law. In some samples therefore 8.6 rather than the usual 4.0 cc. of alcohol were added in order to obtain more suitable conditions for photometry. The measurements for such samples were reduced to terms of a 4.0 cc. dilution by application of Beer's law, and all the following data are given on this basis. In these cases the error in the extinction value introduced by errors in evaluating the plates is not more than 0.06.

Absorption Spectra of Reaction Mixtures

The extinction, represented by the symbol K, is defined by $Kl = \log (i'/i)$, where i' and i represent respectively the intensities of light transmitted through a thickness l of the blank and test solutions. The K-λ curves therefore correspond to the absorption spectra of compounds associated with the presence of the hormones.

No bands were detected in the λ 7000 to 9000 region for any of the reaction mixtures studied; this region has accordingly been omitted from the graphs. On the short wave-length end of the spectrum the observations were limited by the excessive absorption of the reagents. The extinction (against alcohol) of a blank reaction mixture made up with 11.6 mg. per cc. stock dinitrobenzene and developed for 80 minutes at 25.1° was 0.30 at λ 5200, 0.40 at λ 4025, rose gradually to 1.4 at λ 3115, and then rapidly to 3.2 at λ 2950. The absorption of the blank in this region is due almost entirely to the presence of the dinitrobenzene, since alcoholic KOH in the concentration used has an extinction of only 0.1 at λ 2900, 0.2 at λ 2700, and 0.4 at λ 2400. Examination of the spectra of reaction mixtures made up with 11.6 mg. per cc. stock dinitrobenzene was therefore impractical below about λ 3100 with our apparatus. Reduction of the amount of added dinitrobenzene, however, permitted examination of the spectra at shorter wavelengths. The extinction (against alcohol) of a blank reaction mixture made up with 1.74 mg. per cc. stock dinitrobenzene and developed for 100 minutes at room temperature was 0.4 at λ 2950, 0.8 at λ 2800, and 1.0 at λ 2780. Under such conditions the spectra could be examined down to about λ 2700.

Typical data on the extinction curves for reaction mixtures containing androsterone, testosterone, or both, are presented in Figs. 1 to 5. The following remarks may be made concerning the main features of these results.

The absorption spectra of reaction mixtures containing androsterone are quite different from those of mixtures containing testosterone. The spectra of the former are characterized by the appearance of the visible band (maximum at λ 5200), and by a minimum at about λ 3800; those of the latter exhibit low absorption in the λ 5200 region, a band having its maximum at about λ 3800, and strongly increasing absorption toward still shorter wave-lengths (Curves *a* and *b*, Figs. 1 and 2). It may be noted here that an increase in the amount of dinitrobenzene added shifts the strong absorption of the testosterone mixture below λ 3000 toward longer wave-lengths, and obscures the minimum at λ 3200. The spectra are presumably due almost entirely to the hormone compounds, since equivalent alcoholic solutions of the hormones themselves do not produce a comparable extinction. Calculations based on unpublished data show that the extinction of a 0.043 mg. per cc. solution of androsterone in alcohol is only 0.006 at λ 3000, while the corresponding value for testosterone is about 0.03.

The shape of the extinction curve for androsterone mixtures is almost independent of changes in various factors studied (Fig. 3). Under fixed reaction conditions it does not vary with the amount of androsterone present. An increase in the amount of dinitrobenzene added (from 0.4 to 2.3 mg.) possibly results in a slightly greater relative absorption below λ 3500. The curves for mixtures developed for 10 minutes and for 60 minutes differ in shape only in that the former has a slightly lower relative absorption in the λ 3800 region.

On the other hand the shape of the curve for testosterone mixtures is considerably affected by changes in some of the factors studied (Fig. 4). An increase in the amount of dinitrobenzene added (from 0.4 to 2.3 mg.) causes not only the previously mentioned shift toward longer wave-lengths of the strong ultraviolet absorption and the disappearance of the λ 3200 minimum, but also considerably decreases the extinction at λ 3800 relative to that at λ 5200. A decrease in the amount added (from 0.4 to 0.23 mg.) has the opposite effect as might be expected. This change in the spectrum appears more likely to be related to the effect of environment on the absorbing molecules than to any change in the compound formed in the reaction. Under fixed reaction conditions the shape of the curve is little, if at all, affected by changes in the amount of testosterone present, and there is no definite evidence that it is appreciably different for development times of 39 and 101 minutes.

The shape of the curve for mixtures containing both androsterone and testosterone is almost, but not quite, that calculated by superimposing the curves for equivalent amounts of the hormones developed separately; the values of the extinction are, however, somewhat less than those calculated by superposition. These features are illustrated in Curves *c* and *d* of Figs. 1 and 2,

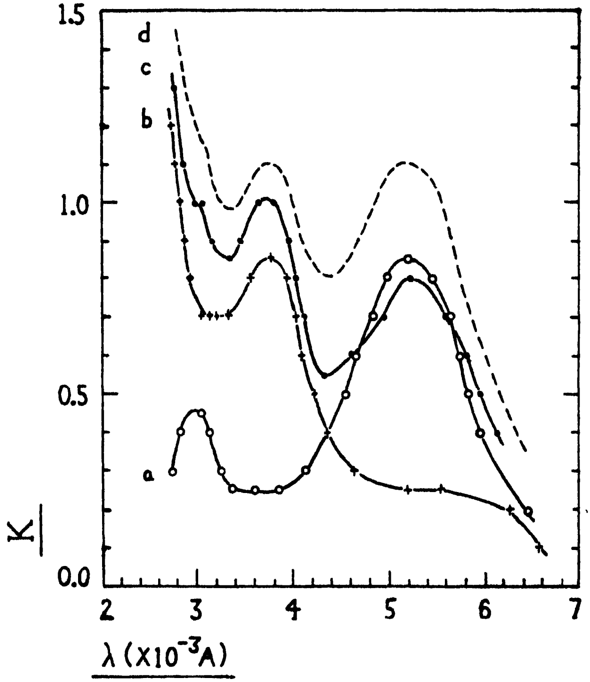


FIG. 1. Extinction curves for androsterone, testosterone, and androsterone plus testosterone reaction mixtures; dinitrobenzene stock solution 2.0 mg. per cc., 80 minutes development at 25.1°. Curve *a* 0.18 mg. of androsterone, Curve *b* 0.19 mg. of testosterone, Curve *c* 0.18 mg. of androsterone plus 0.19 mg. of testosterone, Curve *d* sum of Curves *a* and *b*.

and will be referred to later. The curves of Fig. 5 have been included in this article because they show clearly the effects produced on the absorption curve by increasing the testosterone content of a mixture containing an essentially constant amount of androsterone. It will be noted that as the testosterone content increases, there is a marked increase in the violet and ultraviolet absorption.

Analysis of Solutions of Hormones

It should be emphasized that the present problem differs considerably from that usually encountered in making analyses with the aid of absorption spectra. It is required to determine the concentrations, not of the substances directly responsible for the appearance of the observed bands, but of the hormones from which these substances are produced. A consideration of the laws governing chemical reactions indicates that formation of a compound from one hormone will be influenced by the presence of a second hormone which takes part in a reaction involving the same reagents. Consequently the extinction curve for a reaction

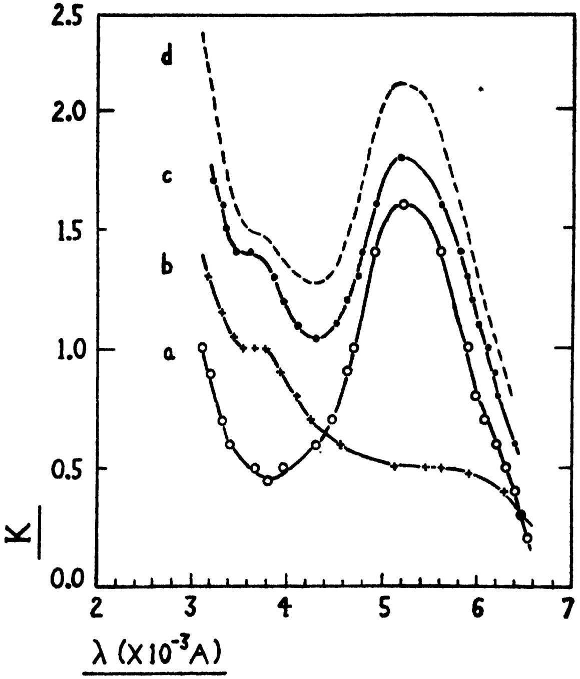


FIG. 2. Extinction curves for androsterone, testosterone, and androsterone plus testosterone reaction mixtures; dinitrobenzene stock solution 11.6 mg. per cc., 80 minutes development at 25.1°. Curve *a* 0.15 mg. of androsterone, Curve *b* 0.19 mg. of testosterone, Curve *c* 0.15 mg. of androsterone plus 0.19 mg. of testosterone, Curve *d* sum of Curves *a* and *b*.

mixture containing two hormones cannot be expected to correspond exactly with that calculated by superimposing the curves for

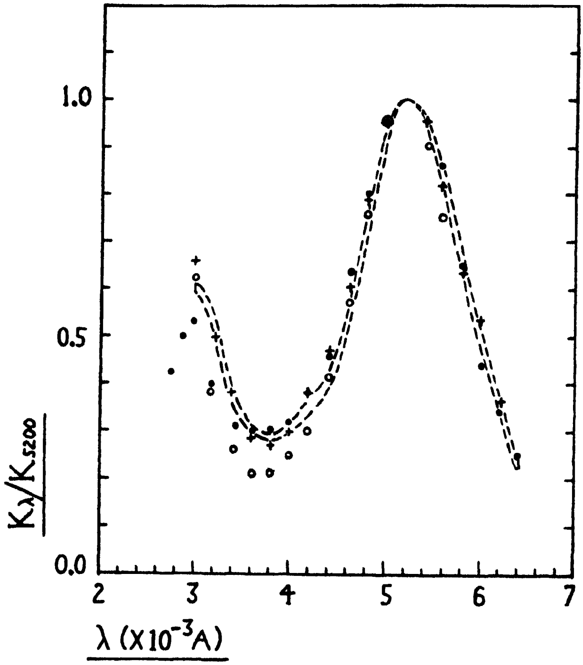


FIG. 3. Effect of various factors on the shape of the extinction curve for androsterone reaction mixtures. All determined points for reaction mixtures containing 0.10, 0.15, 0.30, and 0.40 mg. of androsterone (stock dinitrobenzene, 11.6 mg. per cc., development for 80 minutes at 25.1°) fall within the area enclosed by the dashed lines; the points have not been plotted in order to avoid confusion. Points indicated by ● give the curve shape for a reaction mixture containing 0.18 mg. of androsterone developed for 80 minutes at 25.1°, but made up with 2.0 mg. per cc. stock dinitrobenzene solution. The points indicated by + and ○ give respectively the curve shapes for reaction mixtures containing 0.2 mg. of androsterone made up with 20 mg. per cc. stock dinitrobenzene solution and developed at room temperature for 60 minutes and 10 minutes.

equivalent amounts of the hormones developed separately. If the discrepancy is large, it cannot be disregarded in any method of assay which depends on a determination of light absorption.

The data of Figs. 1 and 2 show that the extinction of a mixture containing both androsterone and testosterone is appreciably lower

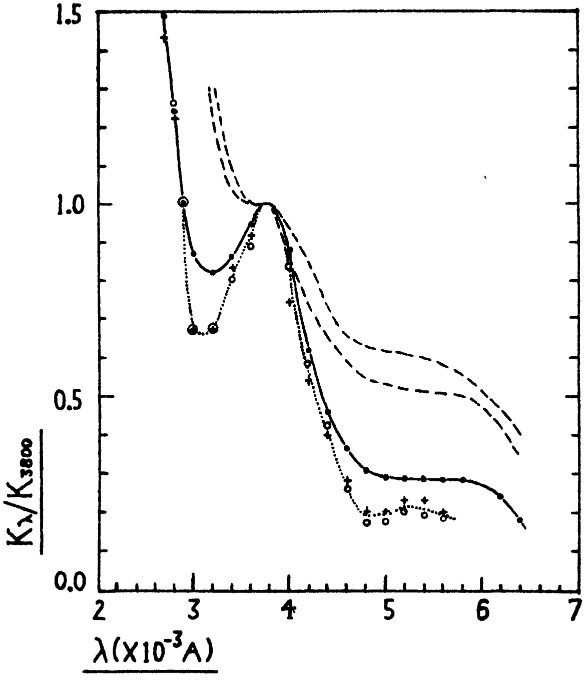


FIG. 4. Effect of various factors on the shape of the extinction curve for testosterone reaction mixtures. All determined points for reaction mixtures containing 0.10, 0.19, 0.30, and 0.40 mg. of testosterone made up with 11.6 mg. per cc. stock dinitrobenzene solution and developed for 80 minutes at 25.1° fall within the area enclosed by the dashed lines; the points have not been plotted in order to avoid confusion. Points indicated by ● give the curve shape for a reaction mixture containing 0.19 mg. of testosterone developed for 80 minutes at 25.1°, but made up with 2.0 mg. per cc. stock dinitrobenzene solution. Points indicated by + and ○ give respectively the curve shapes for reaction mixtures containing 0.2 mg. of testosterone made up with 1.16 mg. per cc. stock dinitrobenzene solution and developed at room temperature for 39 minutes and 101 minutes.

than it would be if the reactions involving the hormones were entirely independent. For the following reasons this decrease is attributed to a reduced formation of the androsterone compound in the presence of testosterone; there is no indication that formation of the testosterone compound is noticeably affected by the presence of androsterone.

On the average, the discrepancy between the observed extinction of a mixture containing both hormones and the value calculated by adding the observed extinctions for equivalent amounts of the hormones developed separately, is about 9 per cent at λ 3800, but increases to about 18 per cent at λ 5200. The testosterone compound is usually responsible for the greater part of the absorption at λ 3800 in these experiments, while the extinction at λ 5200 is mostly due to the androsterone compound. The discrepancies are such as would appear if the amount of the androsterone com-

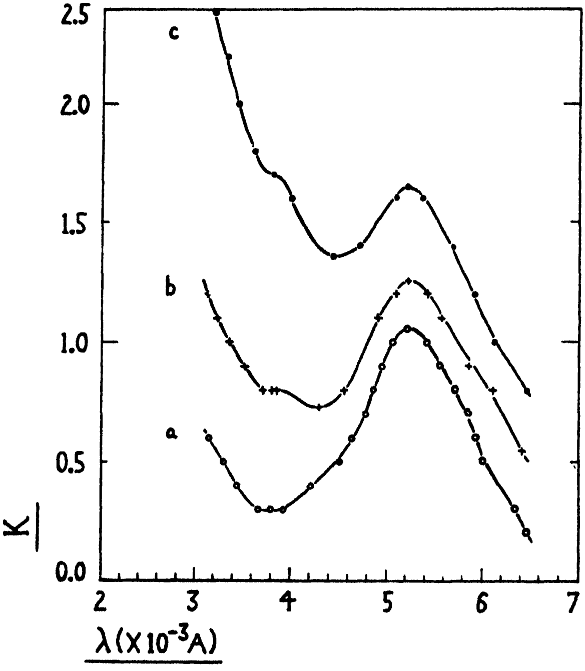


FIG. 5. Effect of testosterone on the extinction curve of reaction mixtures containing androsterone; stock dinitrobenzene solution 11.6 mg. per cc., 80 minutes development at 25.1°. Curve *a* 0.10 mg. of androsterone, no testosterone, Curve *b* 0.11 mg. of androsterone plus 0.10 mg. of testosterone, Curve *c* 0.11 mg. of androsterone plus 0.30 mg. of testosterone.

pound present were less than that expected on the basis of the results obtained with separate development (Figs. 1 and 2 and Table II).

The discrepancy referred to above increased at λ 5200 but was not much affected at λ 3800, when the amount of added dinitrobenzene was reduced from 2.3 to 0.4 mg. This fact furnishes further support for the view that formation of the androsterone compound is noticeably affected by changes in reaction conditions, while the formation of the testosterone compound is not (Figs. 1 and 2).

The calibration curve (*viz.* extinction *versus* amount of hormone, developed separately) for androsterone is not linear; the amount of the compound produced per unit amount of androsterone decreases as the total amount of androsterone present increases. The efficiency of the reaction in producing the compound is therefore influenced even by moderate increases in the amount of androsterone itself. On the other hand the calibration curve for testosterone is linear within the experimental error over the range covered by the experiments; *i.e.*, the efficiency of formation of the testosterone compound is essentially constant. Both these features are in accord with the views expressed in the two paragraphs above. The calibration curves are given in Fig. 6.

Absorption spectrum determinations of the amounts of androsterone and testosterone in synthetic mixtures agree reasonably well with the known values provided the assumption be made that the presence of testosterone affects the androsterone reaction and that the converse is not so (Table II).

It is clear from the preceding discussion that before accurate analysis of mixtures of the hormones can be made one must know (*a*) the calibration curve for each of the hormones, and (*b*) the effect produced by the presence of each hormone on the formation of the absorbing compound from the other. The first of these is readily ascertained; the second must be inferred from appropriate data. On the basis that the presence of androsterone does not affect the testosterone reaction, the figures in the last column of Table I give some indication of the decrease in efficiency of the androsterone reaction due to the presence of testosterone. This decrease appears to depend on the relative amounts of androsterone and testosterone present; it is about 33 per cent when the ratio of the amounts present is 0.37, and about 16 per cent when the ratio is 3.2. Strictly speaking the actual efficiencies of the reactions are expected to be representable by complicated functions involving the amounts of hormones and reagents and factors descriptive of the experimental conditions employed. The term efficiency as used above refers only to the amount of a compound present after a given time of development under given conditions; it is conceivable that alteration of some factor might result in a lowered efficiency in this sense, but at the same time might produce an increased efficiency at some other point in the reaction. It should therefore be emphasized that extrapolation of the findings to other regions or experimental conditions is dangerous.

Table I

Relation between Observed and Expected Extinction for Reaction Mixtures Containing both Hormones

The symbols have the following significance; $\bar{K}$ denotes the extinction of the mixture containing both hormones, K the extinction of the androsterone compound when testosterone is absent, and K_A the extinction of the androsterone compound when testosterone is present.

Androsterone	Testosterone	Ratio	$\bar{K}$(observed)		$\bar{K}$(expected)*		$\frac{\bar{K}\text{(observed)}}{\bar{K}\text{(expected)}}$		K_A (calculated)†		Average $\frac{K_A}{K}$
			λ 5200	λ 3800	λ 5200	λ 3800	λ 5200	λ 3800	λ 5200	λ 3800	
mg.	*mg.*										
0.11	0.30	0.37	1.66	1.70	1.97	1.84	0.84	0.92	0.87	0.21	0.67
0.22	0.31	0.71	2.26	2.00	3.06	2.17	0.73	0.92	1.45	0.47	0.69
0.15	0.19	0.79	1.80	1.32	2.14	1.44	0.84	0.92	1.26	0.33	0.76
0.11	0.10	1.10	1.25	0.80	1.50	0.93	0.83	0.86	0.93	0.22	0.71
0.32	0.21	1.5	2.90	1.80	3.64	1.96	0.79	0.92	2.31	0.72	0.79
0.32	0.10	3.2	2.96	1.30	3.37	1.46	0.88	0.89	2.64	0.72	0.84

* The sum of the extinctions for equivalent amounts of the hormones developed separately.

† The extinction for the mixture minus that for the testosterone compound developed separately; this calculation assumes that the amount of testosterone compound formed is the same whether androsterone is present or not.

For the purposes of the present paper the relative efficiency of the androsterone reaction in the presence of testosterone is considered to have a constant value equal to the average of the figures given in the last column of Table I; *i.e.*, the amount of androsterone compound formed in the stated time under the stated conditions is taken to be 75 per cent of that which would have been formed had the testosterone been absent. The calibration curves for the hormones are well represented by the following empirical equations (see Fig. 6).

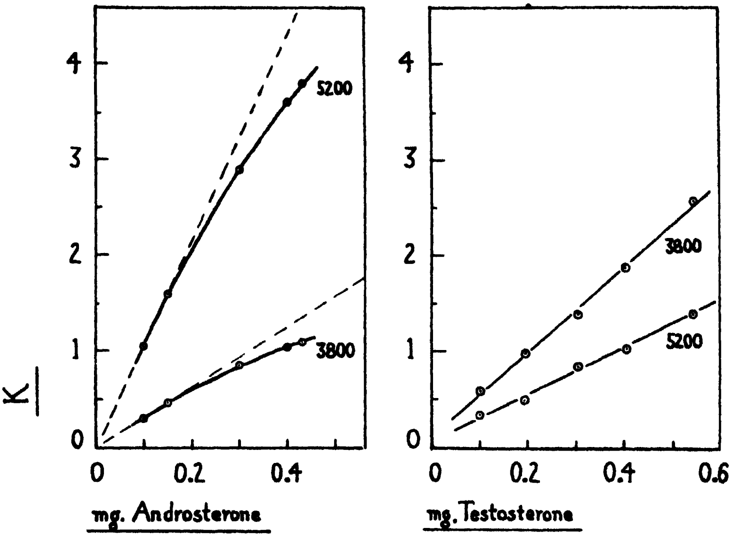


Fig. 6. Calibration curves for androsterone and testosterone showing the relation between the extinction at λ 5200 or 3800 and the amount of hormone present; the hormones were developed separately.

$$\begin{aligned} K_{5200} &= 11.0M - 5.0M^2 \\ K_{3800} &= 3.14M - 1.43M^2 \\ k_{5200} &= 2.51m + 0.04 \\ k_{3800} &= 4.50m + 0.08 \end{aligned} \qquad (1)$$

In these equations K and k represent respectively the extinction due to the androsterone compound and to the testosterone compound; M and m denote respectively the amounts (in mg.) of androsterone and testosterone initially present. On the basis outlined above the extinction due to the androsterone compound in mixtures containing testosterone is given by equations obtained from the first two by multiplying the coefficients by 0.75. Accordingly, for such mixtures the total extinction is given by

$$\begin{aligned} \bar{K}_{5200} &= 8.25M - 3.75M^2 + 2.51m + 0.04 \\ \bar{K}_{3800} &= 2.35M - 1.07M^2 + 4.50m + 0.08 \end{aligned} \qquad (2)$$

Solution of these equations leads to

$$m = \frac{3.52\bar{K}_{3800} - (\bar{K}_{5200} + 0.24)}{13.3} \qquad (3)$$

$$M = 1.10 - (1.20 + 0.174\bar{K}_{3800} - 0.316\bar{K}_{5200})^{\frac{1}{2}}$$

It is possible to calculate M and m from these equations once $\bar{K}_{5200}$ and $\bar{K}_{3800}$ have been determined from examination of the absorption spectrum.

A comparison of determined and known amounts of androsterone and testosterone in alcoholic solutions is given in Table II. The values determined with the aid of Equations 3 are given in Columns 5 and 6. Since it is of some interest to know the effect of neglect of the influence of testosterone on the androsterone reaction, values determined with the aid of Equations 1 only are included in Columns 7 and 8. Column 9 contains determinations in terms of "sterone" units (8), obtained by reading off from the androsterone calibration curve the amount of androsterone which would give an equivalent extinction at λ 5200.

The following remarks may be made concerning the data of Table II. (*a*) The determined values of Columns 5 and 6 agree quite well with the known values. (*b*) Determinations made on the assumption that the reactions are independent (Columns 7 and 8) are somewhat lower than the known values for androsterone. (*c*) The "sterone" assay gives values for these samples which are in fairly good agreement with the known androsterone contents. At first sight it is rather surprising that this should be so, since the extinction at λ 5200 is partly due to the testosterone compound. The explanation is that *for these samples* the additional extinction due to the testosterone compound is partially compensated for by a reduced efficiency of the androsterone reaction.

It is of some interest to consider how the spectrochemical assay and the "sterone" assay of these samples would be expected to compare with the biological assay. (The term spectrochemical is used to denote a method which involves both spectroscopic and chemical treatment.) In view of the fact that testosterone is about 6 times more active physiologically than androsterone, the "equivalent" mg. of androsterone contained in each of the samples is obtained by adding the androsterone content to 6 times the testosterone content. The figures of Table III were obtained in this way from the data of Table II; the values for the biological assay were calculated from the known composition of the samples.

Table II

Comparison of Determined and Known Composition of Synthetic Hormone Solutions

Known composition		Observed $\bar{K}$		Determined composition				
Androsterone (1)	Testosterone (2)	λ 5200 (3)	λ 3800 (4)	Androsterone (5)	Testosterone (6)	Androsterone (7)	Testosterone (8)	"Sterone" units (9)
mg.	mg.			mg.	mg.	mg.	mg.	
0.11	0.10	1.25	0.80	0.12	0.10	0.08	0.10	0.12
0.11	0.30	1.66	1.70	0.11	0.31	0.08	0.31	0.16
0.15	0.19	1.80	1.32	0.16	0.20	0.12	0.20	0.17
0.32	0.10	2.96	1.30	0.39	0.10	0.27	0.10	0.31
0.32	0.21	2.90	1.80	0.32	0.24	0.23	0.24	0.30
0.22	0.31	2.26	2.00	0.19	0.34	0.13	0.34	0.22

Table III

Comparison of Activities of Synthetic Samples As Assayed by Different Methods (Estimated)

The results are given as equivalent mg. of androsterone.

Biological assay	Spectrochemical assay	"Sterone" assay
0.71	0.72	0.12
1.91	1.97	0.16
1.29	1.36	0.17
0.92	0.99	0.31
1.58	1.76	0.30
2.08	2.23	0.22

The data of Table III indicate that application of spectrochemical methods to the assay of urine extracts may be expected to lead to more significant results than do present methods. While testosterone has not yet been isolated from the human urine, there is the possibility that it may sometimes be present in relatively high concentrations. The failure of present methods of "sterone" assay to give a reliable indication of the activity of urine extracts which possess marked absorption in the violet region may possibly be of some significance in this respect. Difficulties are to be anticipated in the application of spectrochemical methods to the assay of urine extracts: the inactive substances present may not only give rise to interfering spectra, but may also seriously influence the chemical reactions which result in the formation of the absorbing compounds. The findings of the present investigation give some basis for hope, however, that more satisfactory methods of assay can be developed. Work on the problem is now in progress.

This work was carried out under a grant from the Rockefeller Foundation to Professor George R. Harrison for the application of spectrophotometry to medical problems.

SUMMARY

The compounds formed by androsterone and testosterone in the *m*-dinitrobenzene reaction have distinctly different absorption spectra in the wave-length region λ 2800 to 7000. These differences permit reasonably accurate determinations to be made of the amounts of synthetic androsterone and testosterone in alcoholic solutions containing both hormones. There is evidence that the reaction involving androsterone is appreciably influenced by the presence of testosterone; for the most accurate determinations it is necessary to take this factor into consideration. It is hoped that an application of these findings to the androgen assay of urine extracts may yield results of greater significance than do present methods. A study of the problem is now in progress.

BIBLIOGRAPHY

1. Koch, F. C., *Phys〈iol. Rev.*, **17**, 153 (1937).
2. Oppenauer, R., *Tab. Biol.*, **15**, 13 (1938).
3. Zimmerman, W., *Z. physiol. Chem.*, **233**, 257 (1935).
4. Zimmerman, W., *Z. physiol. Chem.*, **245**, 47 (1936).
5. Oesting, R. B., *Proc. Soc. Exp. Biol. and Med.*, **36**, 524 (1937).
6. Oesting, R. B., and Webster, B., *Endocrinology*, **22**, 307 (1938).
7. Wu, H., and Chou, C.-Y., *Chinese J. Physiol.*, **11**, 413, 429 (1937).
8. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, **32**, 1312 (1938).
9. Neustadt, R., *Endocrinology*, **23**, 711 (1938).

INDEX TO AUTHORS

A

Addis, T. See Poo, Lew, and Addis, 69

Adlersberg, David, and Ellenberg, Max. Effect of carbohydrate and fat in the diet on uric acid excretion, 379

Allinson, M. J. Carl. See Miller, Baker, and Allinson, lxx

Ames, James B. See Ratish, Bullowa, Ames, and Scudi, 279

Andersch, Marie, and Fay, Marion. The biochemistry of strontium, iii

Andrews, James C., and Andrews, Kathleen Crandall. Studies on cystinuria; the effect of diuresis on the excretion of cystine by the cystinuric, iii

—. See Kyker and Andrews, lviii

Andrews, Kathleen Crandall. See Andrews and Andrews, iii

Arnold, Aaron. Influence of the composition of the diet on the thiamine requirement of dogs, iv

B

Babbitt, Dorothea. See Bratton and Marshall, 537

Bachman, Carl. See Gurin, Bachman, and Wilson, 525, xxxvii

Baer, Erich, and Fischer, Hermann O. L. Studies on acetone-glyceraldehyde. IV. Preparation of $d(+)$-acetone glycerol, 463

— and —. V. Synthesis of optically active glycerides from $d(+)$-acetone glycerol, 475

— and —. VI. Synthesis of the biological $l(-)$-α-glycerophosphoric acid, 491

Baker, Zelma. See Miller, Baker, and Allinson, lxx

Ball, Eric G. Xanthine oxidase: purification and properties, 51

Bandes, Joseph. See Hollander and Bandes, xlvii

Barron, E. S. Guzman. See Lyman and Barron, lxii

Bates, Robert W., and Riddle, Oscar. The question of a sex difference in crop sac response to prolactin, v

Baumann, C. A., Jacobi, H. P., and Rusch, H. P. The effect of diet on tumors produced in various ways, vi

Baumann, Emil J., and Sprinson, David B. Interrelation of the thyroid and parathyroid glands, vi

Beach, Eliot F., Erickson, Betty Nims, Bernstein, Samuel S., Williams, Harold H., and Macy, Icie G. The amino acid composition of erythrocyte posthemolytic residue of five mammalian species, 339
—. See Hoffman and Beach, xlv
Beard, Howard H., Koven, A. Link, and Pizzolato, Philip. The biological relation of creatine and creatinine, vii
—. See Koven and Beard, lvi
Beeson, Paul B. See Goebel, Beeson, and Hoagland, xxxiv
Belfer, Samuel. See Eder, Bradley, and Belfer, 551, xxiii
Bergmann, Max, and Stein, William H. A new principle for the determination of amino acids, and its application to collagen and gelatin, 217
Bernhart, F. W. Denaturation and molecular splitting of egg albumin by ultraviolet radiant energy, 289
Bernheim, Frederick, and Bernheim, Mary L. C. Note on the action of manganese and some other metals on the oxidation of certain substances by liver, 79
Bernheim, Mary L. C. See Bernheim and Bernheim, 79
Bernstein, Samuel S. See Beach, Erickson, Bernstein, Williams, and Macy, 339
Bibler, Walter G. See Eichelberger and Bibler, xxiv
Bischoff, Fritz, Long, M. Louisa, and Rupp, J. Jerome. Endocrine factors influencing tumor development, viii
Blanchard, Muriel H. See Ferry, Cohn, Oncley, and Blanchard, xxviii
Block, Richard J. The composition of keratins. The amino acid composition of hair, wool, horn, and other eukeratins, 181
— and **Bolling, Diana.** Chemical reactions involved in the determination of phenylalanine, viii
Bloor, W. R. The determination of adrenalin in blood, ix
Bodansky, Oscar. Effect of temperature on activity of bone phosphatase, x
Bolling, Diana. See Block, 181
—. See Block and Bolling, viii
Bollman, Jesse L. See Flock and Bollman, xxx
—. See Flock, Hester, and Bollman, 153
Bonsnes, Roy W. See White and Bonsnes, cix
Borsook, Henry, and Dubnoff, Jacob. The biological synthesis of hippuric acid *in vitro*, cxiv
Bradley, H. C. See Eder, Bradley, and Belfer, 551, xxiii
Brand, Elliott. See Brodie, Leshin, and Brand, xi
Brand, Erwin, Kassell, Beatrice, and Heidelberger, Michael.

On the structure of thyroglobulin, xi

Brand, Erwin. See Kassell, Pearlstein, Brand, and Cahill, p. 1

Brand, Florence C. See Block, 181

—. See Sperry and Brand, xcvii

Bratton, A. Calvin, and **Marshall, E. K., Jr.** A new coupling component for sulfanilamide determination, 537

Bridwell, E. G. See Harger, Bridwell, and Raney, xxxviii

Brodie, Bernard B., Leshin, S., and **Brand, Elliott.** The use of bromide as a measure of extracellular fluids, xi

Browne, J. S. L. See Venning, Weil, and Browne, cvii

Bruger, Maurice. See Member and Bruger, lxix

Bryan, W. Ray. See Knudson, Sturges, and Bryan, 721

Bullowa, Jesse G. M. See Ratish, Bullowa, Ames, and Scudi, 279

Butler, A. W. See Dunn, Butler, and Frieden, xxii

C

Cahill, George F. See Kassell, Pearlstein, Brand, and Cahill, p. 1

Cahill, William M. Racemization of amino acids and dipeptides on acetylation with ketene, xii

Callan, Thomas P. See Toennies and Callan, civ

Carr, C. Jelleff, and **Forman, Sylvan E.** Sugar alcohols. XX. The fate of *d*-sorbitol, styracitol, and *l*-sorbose in the animal body, 425

Carter, Herbert E., Osman, Elizabeth, Levine, Harold, and **Gamm, Stanford.** Metabolism of fatty acids, xiii

Ceithaml, Joseph. See Evans, Ceithaml, Hines, and Koch, xxvi

Cerecedo, Leopold R. See Sullivan and Cerecedo, c

Chaikoff, I. L. See Jones, Chaikoff, and Lawrence, 631

—. See Montgomery, Entenman, and Chaikoff, 387

—. See Perlman and Chaikoff, 735

Chandler, Joseph P. See du Vigneaud, Chandler, Moyer, and Keppel, cviii

Chargaff, Erwin. The determination of radioactive isotopes in organic material, xiii

—. The relative speed of formation of lecithin and cephalin in the body, xiv

—. Unstable isotopes. I. The determination of radioactive isotopes in organic material, 579

—. II. The relative speed of formation of lecithin and cephalin in the body, 587

Christman, Clarence C. See Tipson, Christman, and Levene, 609

Clemence, Leroy W. See Raiziss, Severac, Moetsch, and Clemence, lxxxi

Clowes, G. H. A., Keltch, A. K., and Krahl, M. E. The ionization of barbituric acids and anesthetic bases in relation to their anesthetic action, xv

Cohn, David J., Tannenbaum, Albert, Thalhimer, William, and Hastings, A. Baird. Influence of oxygen and carbon dioxide on the blood of normal and pneumonic dogs, 109

Cohn, Edwin J. See FERRY, COHN, ONCLEY, and BLANCHARD, xxviii

Cohn, Waldo E., and Greenberg, David M. Studies in mineral metabolism with the aid of artificial radioactive isotopes. III. The influence of vitamin D on the phosphorus metabolism of rachitic rats, xvi

—. See JOSEPH, COHN, and GREENBERG, 673

Cole, Versa V. See HARNED and COLE, xxxix

Connolly, Mary K. See FERRY, FERRY, and CONNOLLY, xxix

Cook, Gladys M. See MITCHELL, COOK, and O'BRIEN, lxxi

Coolidge, Thomas B. The van den Bergh reaction, xvii

Corley, Ralph C., and Lyons, Mary. Formation of glycogen from amino and alkyl derivatives of the fatty acids, xvii

Craig, Lyman C. See JACOBS and CRAIG, 431, 715

—. See JACOBS, ELDERFIELD, and CRAIG, 439

Cretcher, Leonard H. Structure and antipneumococcic activity in the cinchona series, xviii

Cuthbert, F. P. See GRAY, IVY, and CUTHBERT, 173

D

Daft, Floyd Shelton. See SEBRELL and DAFT, lxxxix

Daly, C. See DILL, JOHNSON, and DALY, xx

Dann, W. J. The incidence of acrodynia among rats receiving diets deficient in vitamin B_6, xviii

Daoust, Robert. See MCCULLAGH and DAOUST, lxv

Dawson, Martin H. See MEYER, SMYTH, and DAWSON, 319, lxx

Deuel, Harry J., Jr., Hallman, Lois F., and Quon, S. A simple procedure of the study of fat absorption, xix

Dill, D. B., Johnson, R. E., and Daly, C. Metabolic effects of adrenalin and benzedrine, xx

Dimick, Mildred K. See SMITH and DIMICK, xciii

Dittebrandt, Marlowe. See TODD, MONTAGUE, DITTEBRANDT, and WEST, ciii

Dorfman, Albert, Horwitt, M. K., Koser, S. A., and Saunders, Felix. The use of the dysentery organism for the quantitative determination of nicotinic acid, xx

Doster-Virtue, Mildred E. See VIRTUE and DOSTER-VIRTUE, 665

Drabkin, David L., and **Singer, Richard B.** The effect of light scattering upon the absorption spectra of non-hemolyzed erythrocytes, xxi

Driver, Robert L. See SEALOCK, ZIEGLER, and DRIVER, lxxxix

Dubnoff, Jacob. See BORSOOK and DUBNOFF, cxiv

DuFour, Emma H. See SEIBERT and DUFOUR, xc

Dunn, Max S., **Butler, A. W.**, and **Frieden, Edward H.** The function of carbonate in the synthesis of glycine from chloroacetic acid, ammonium hydroxide, and ammonium carbonate, xxii

Dyer, Helen M. See KIES, DYER, WOOD, and DU VIGNEAUD, 207

E

Eakin, Robert E., and **Williams, Roger J.** Biotic acid; a new growth substance in liver, xxiii

Eck, John C., and **Thomas, Byron H.** The chemical activation of sterols. V. A study of the relationship between chemical activation and configuration of various sterols and derivatives, 257

— and —. VI. A study of various reagents in chemical activation and in sterol color reactions, 267

Eckstein, H. C. See TREADWELL and ECKSTEIN, 373, cv

Eder, Howard, **Bradley, H. C.**, and **Belfer, Samuel.** The survival of cathepsin, xxiii

—, —, and —. The survival of cathepsin in autolysis, 551

Edson, M. See HEARD and EDSON, xli

Eichelberger, Lillian. The distribution of body water in skeletal muscle in dogs with impaired renal function, 137

— and **Bibler, Walter G.** Water and electrolyte content of dog kidneys, xxiv

Elderfield, Robert C. See JACOBS, ELDERFIELD, and CRAIG, 439

Ellenberg, Max. See ADLERSBERG and ELLENBERG, 379

Elvehjem, C. A. See FROST and ELVEHJEM, 23, xxxi

—. See KOHLER, ELVEHJEM, and HART, 501

Embree, Norris D. The occurrence of cyclized vitamin A in fish liver oils, 187

Endicott, Elizabeth E. See WILLIAMS, SHEPHERD, and ENDICOTT, cxi

Engel, R. W., and **Phillips, Paul H.** Chemical and histologic studies of fatty livers produced in thiamine therapy of experimental vitamin B_1 deficiency, xxv

Entenman, C. See MONTGOMERY, ENTENMAN, and CHAIKOFF, 387

Erickson, Betty Nims. See BEACH, ERICKSON, BERNSTEIN, WILLIAMS, and MACY, 339

Evans, E. A., Jr. The influence of spermine and spermidine on tissue oxidations, xxv

Evans, John S., Ceithaml, Joseph, Hines, L. R., and Koch, F. C. On the mechanism of augmentation of gonadotropic activity, xxvi

Evelyn, Kenneth A., and Malloy, Helga Tait. The determination of pH with the photoelectric colorimeter, xxvii

Everett, Mark R. See SHEPPARD and EVERETT, xci

Exton, William G. See ROSE, SCHATTNER, and EXTON, lxxxiv

F

Fay, Marion. See ANDERSCH and FAY, iii

Feinstein, R. N., and Stare, F. J. Thermostable oxidations in tumor tissue, xxviii

Fenn, W. O. The deposition of potassium and phosphate with glycogen in rat livers, 297

Ferry, J. D., Cohn, Edwin J., Oncley, J. L., and Blanchard, Muriel H. The dielectric properties of lactoglobulin and its interaction with ions and dipolar ions, xxviii

Ferry, Ronald M., Ferry, Virginia T., Jr., and Connolly, Mary K. Studies on the proteins of the kidney. I, xxix

Ferry, Virginia T., Jr. See FERRY, FERRY, and CONNOLLY, xxix

Fevold, H. L. Chemical differences of the follicle-stimulating and luteinizing hormones of the pituitary, 83

Field, Henry, Jr. See MELNICK and FIELD, lxviii

Findlayson, Joyce. See GIBSON, FINDLAYSON, and PAUL, xxxiii

Fischer, Hermann O. L. See BAER and FISCHER, 463, 475, 491

Flock, Eunice V., and Bollman, Jesse L. Carbohydrate changes in working muscle, xxx

—, **Hester, Harold R., and Bollman, Jesse L.** Phospholipid changes during the production of fatty livers in geese, 153

Forman, Sylvan E. See CARR and FORMAN, 425

Friedell, H. L. See KEYS and FRIEDELL, li

Frieden, Edward H. See DUNN, BUTLER, and FRIEDEN, xxii

Froeschle, Paul F., and Pierce, H. B. Gastric emptying of cereal starch, xxxi

Frost, D. V., and Elvehjem, C. A. Factor W and its relation to the vitamin B complex, 23

— and —. The rôle of iron, copper, and cobalt in hemoglobin production in dogs on a milk diet, xxxi

Fuller, Henry S. See HITCHINGS and FULLER, xlv

G

Gaebler, Oliver Henry, and Zimmerman, Walter J. Effects of an anterior pituitary growth preparation on metabolism in phlorhizin diabetes, xxxii

Gamm, Stanford. See Carter, Osman, Levine, and Gamm, xiii

Gavin, Gertrude. See McHenry and Gavin, 45, lxvi

Gersdorff, Charles E. F. See Jones and Gersdorff, xlix

Gibson, R. B., Findlayson, Joyce, and **Paul, W. D.** Renal threshold for glucose in pregnancy, xxxiii

Glick, David. Specificity of choline esterase, xxxiv

Goebel, Walther F., Beeson, Paul B., and **Hoagland, Charles L.** The capsular polysaccharide of Type XIV pneumococcus and its relationship to the specific substances of human blood, xxxiv

Goerner, Alfred, and **Goerner, M. Margaret.** Vitamin A and liver cell tumors, 559

Goerner, M. Margaret. See Goerner and Goerner, 559

Goettsch, Marianne, Lonstein, Ida, and **Hutchinson, John J.** Muscle phosphorus in nutritional muscular dystrophy in rabbits, 9

Gordon, Edgar S. See Kimble and Gordon, lii

Goudsmit, Arnoldus, Jr. See Power, Goudsmit, and Keith, lxxix

Graff, Samuel. See Rittenberg, Keston, and Graff, lxxxiii

Gray, J. S., Ivy, A. C., and **Cuthbert, F. P.** The conversion of protein to glucose in depancreatized and phlorhizinized dogs, 173

Greaves, Vera. See Okey and Greaves, lxxvi

Green, Lowell F., McCarthy, James F., and **King, C. G.** Inhibition of respiration and photosynthesis in *Chlorella pyrenoidosa* by organic compounds that inhibit copper catalysis, 447

—. See McCarthy, Green, and King, 455

Greenberg, David M. See Cohn and Greenberg, xvi

—. See Joseph, Cohn, and Greenberg, 673

Greenstein, Jesse P. Studies of multivalent amino acids and peptides. XI. The synthesis of diglycyl-*l*-cystine, 241

—. Sulfhydryl groups in proteins, xxxv

—. Sulfhydryl groups in proteins. II. Edestin, excelsin, and globin in solutions of guanidine hydrochloride, urea, and their derivatives, 233

— and **Klemperer, Friedrich W.** Aspartylhistidine, 245

Griffith, Wendell H. The relation of growth, dietary protein, and liver fat in young rats, xxxvi

Guerrant, Ralph E., and **Hogan, Albert G.** Effect of amino acids on anemia caused by deaminized casein, 363

Gurin, Samuel, Bachman, Carl, and **Wilson, D. Wright.** The carbohydrate of the gonadotropic hormone of pregnancy urine, xxxvii

—, —, and —. The gonadotropic hormone of urine of pregnancy. I. A simple method of extraction and purification, 525

H

Hall, James M. See Roe and Hall, 329

Hallman, Lois F. See Deuel, Hallman, and Quon, xix

Hammett, Frederick S. A property of the hydroxy group in amino acids, xxxvii

Hanes, Frederick M. See Reiser and Hanes, lxxxii

Hanke, Martin E. An oil manometer for the fifteen-fold increase in sensitivity of the manometric gas apparatus of Van Slyke and Neill, xxxviii

Harger, R. N., Bridwell, E. G., and **Raney, B. B.** An aerometric method for the rapid determination of alcohol in water and body fluids, xxxviii

Harned, Ben K., and **Cole, Versa V.** Evidence of hyperfunction of the anterior pituitary in a strain of rats, xxxix

Harris, Milton. See Steinhardt and Harris, xcix

Harris, Philip L., and **Remington, Roe E.** The effect of vitamin A and carotene on goiter due to low iodine intake, xl

Harris, T. N. See Smythe and Harris, xciv

Hart, E. B. See Kohler, Elvehjem, and Hart, 501

Hastings, A. Baird. See Cohn, Tannenbaum, Thalhimer, and Hastings, 109

Heard, R. D. H., and **Edson, M.** Hydrolysis of the conjugated estrogens of pregnant mare urine, xli

Heidelberger, Michael. See Brand, Kassell, and Heidelberger, xi

Heller, Carl G. See Heller, Heller, and Sevringhaus, xli

Heller, Emily J., Heller, Carl G., and **Sevringhaus, Elmer L.** Gonadotropic hormone: assays of human male urine, xli

Helmer, O. M., and **Page, Irvine H.** Purification of renin, xlii

Hendricks, Jeanette B. See Morgan, Hendricks, and Shimotori, lxxii

Hendrickson, Alma R. See Bratton and Marshall, 537

Henny, George C. See Spiegel-Adolf and Henny, xcvii

Hess, W. C., and **Sullivan, M. X.** The acid sensitivity of cystine peptides and anhydrides, xliii

— and —. The cystine content of deaminized proteins, 93

Hester, Harold R. See Flock, Hester, and Bollman, 153

Hickman, K. The nature of vitamin A in fish liver oils, and the need for a new international standard, xliii

Hill, Elsie. See Koehler, Marsh, and Hill, liii

—. See Koehler, Windsor, and Hill, liv

Hill, Robert M., and **Schultz, Fred.** The cause of the fall in body temperature of "immobilized" rats exposed to low environmental temperatures, xliv

Hines, L. R. See EVANS, CEITHAML, HINES, and KOCH, xxvi

Hitchings, George H., and **Fuller, Henry S.** The hydrolysis of adenosinetriphosphate by bone and venom phosphatases, xlv

Hoagland, Charles L. See GOEBEL, BEESON, and HOAGLAND, xxxiv

Hodge, Harold Carpenter. Solubility studies on the calcium phosphates, xlv

Hoffman, Olive D., and **Beach, Eliot F.** Further studies of the posthemolytic residue of erythrocytes. On the chemical composition of the stroma of erythrocytes in pathological conditions, with special attention to polycythemia in man, xlv

Hogan, Albert G., and **Parrott, E. M.** Anemia in chicks due to vitamin deficiency, xlvi

—. See GUERRANT and HOGAN, 363

Hogness, T. R. See HORECKER, STOTZ, and HOGNESS, 251

Hollander, Franklin, and **Bandes, Joseph.** Errors in the dilution indicator technique of gastric analysis, xlvii

Horecker, B. L., **Stotz, Elmer**, and **Hogness, T. R.** The promoting effect of aluminum, chromium, and the rare earths in the succinic dehydrogenase-cytochrome system, 251

Horwitt, M. K. See DORFMAN, HORWITT, KOSER, and SAUNDERS, xx

Howard, R. Palmer. See THORN and HOWARD, cii

Howell, Stacey F. The determination of the urea in chicken blood, 573

Hutchinson, John J. See GOETTSCH, LONSTEIN, and HUTCHINSON, 9

I

Irreverre, Filadelfo. See SULLIVAN and IRREVERRE, ci

Ivy, A. C. See GRAY, IVY, and CUTHBERT, 173

J

Jacobi, H. P. See BAUMANN, JACOBI, and RUSCH, vi

Jacobs, Walter A., and **Craig, Lyman C.** Delphinine. II. On oxodelphinine, 431

— and —. The ergot alkaloids. XVII. The dimethylindole from dihydrolysergic acid, 715

—, **Elderfield, Robert C.**, and **Craig, Lyman C.** The aconite alkaloids. II. The formula of oxonitine, 439

Jellinek, E. Morton, and **Looney, Joseph M.** Statistics of some biochemical variables on healthy men in the age range of twenty to forty-five years, 621

Jensen, H. See TOLKSDORF and JENSEN, cv

Johlin, J. M. The progressive attenuation of insulin by interfacial adsorption, xlviii

Johnson, R. E., and **Wilson, J. W.** Lactic acid as a component of the physicochemical system of human blood, xlviii

—. See DILL, JOHNSON, and DALY, xx

Jones, D. Breese, and Gersdorff, Charles E. F. The effect of storage on the proteins of seeds and their flours. Soy beans and wheat, xlix

Jones, H. B., Chaikoff, I. L., and Lawrence, John H. Radioactive phosphorus as an indicator of phospholipid metabolism. VI. The phospholipid metabolism of neoplastic tissues (mammary carcinoma, lymphoma, lymphosarcoma, Sarcoma 180), 631

Joseph, Michael, Cohn, Waldo E., and Greenberg, David M. Studies in mineral metabolism with the aid of artificial radioactive isotopes. II. Absorption, distribution, and excretion of potassium, 673

Jukes, Thomas H. Experiments with the filtrate factor, 35

K

Kassell, Beatrice, Pearlstein, Leonard, Brand, Erwin, and Cahill, George F. Sulfanilamide metabolism in cystinuria, p. 1

—. See BRAND, KASSELL, and HEIDELBERGER, xi

Keeler, C. E. See TRIMBLE and KEELER, cvi

Keith, Norman M. See POWER, GOUDSMIT, and KEITH, lxxix

Keltch, A. K. See CLOWES, KELTCH, and KRAHL, xv

Kendall, Edward C. A quantitative study of the effect of compounds with cortin-like activity, li

Keppel, Dorothy M. See DU VIGNEAUD, CHANDLER, MOYER, and KEPPEL, cviii

Keston, Albert S. See RITTENBERG, KESTON, and GRAFF, lxxxiii

—. See RITTENBERG, SCHOENHEIMER, and KESTON, 603

Keys, Ancel, and Friedell, H. L. Cardiac output as measured by roentgenkymography, li

Kies, Marian Wood, Dyer, Helen M., Wood, John L., and du Vigneaud, Vincent. A study of the utilization of the optical isomers of N,N′-dimethylcystine, 207

Kimble, Marian S., and Gordon, Edgar S. The importance of riboflavin and ascorbic acid in the utilization of vitamin A, lii

King, C. G. See GREEN, McCARTHY, and KING, 447

—. See LONGENECKER, MUSULIN, and KING, lx

—. See McCARTHY, GREEN, and KING, 455

Klein, J. R. Effect of thyroid feeding and thyroidectomy on the oxidation of amino acids by rat kidney and liver, 659

Klemperer, Friedrich W. See GREENSTEIN and KLEMPERER, 245

Kniazuk, Michael. See WEBB and KNIAZUK, 511

Knott, Elizabeth M. See SHUKERS, KNOTT, and SCHLUTZ, xcii

Knudson, Arthur, Sturges, Stuart, and Bryan, W. Ray. Cholesterol content of skin, blood, and tumor tissue in rats irradiated with ultraviolet light, 721

Koch, F. C. See Evans, Ceithaml, Hines, and Koch, xxvi

Koehler, Alfred E., Marsh, Norman, and Hill, Elsie. The determination of added maltose in the presence of glucose in blood, tissue, and urine, liii

—, **Windsor, Emanuel, and Hill, Elsie.** Maltose utilization in diabetes mellitus, liv

Kohler, G. O., Elvehjem, C. A., and Hart, E. B. The relation of pyrrole-containing pigments to hemoglobin synthesis, 501

—, **Randle, S. B., and Wagner, J. R.** The grass juice factor, lv

Kolb, Joseph J. See Toennies and Kolb, 399

Kondritzer, Albert A. Chemical studies in mental disease. The state of the serum proteins, lv

Koser, S. A. See Dorfman, Horwitt, Koser, and Saunders, xx

Koven, A. Link, and Beard, Howard H. Creatine-creatinine metabolism and the hormones. II. Progestin and antuitrin-T, lvi

—. See Beard, Koven, and Pizzolato, vii

Krahl, M. E. Apparent dissociation constants of thirty substituted barbituric acids as a function of ionic strength and temperature, lvii

—. See Clowes, Keltch, and Krahl, xv

Kramer, Benjamin. See Sobel, Yuska, Petrovsky, and Kramer, xcvi

Kyker, Granvil C., and Andrews, James C. The determination of quinine in blood, lviii

L

Langstroth, G. O., and Talbot, N. B. The absorption spectra of the compounds formed by androsterone and testosterone in the *m*-dinitrobenzene reaction, 759

Lawrence, John H. See Jones, Chaikoff, and Lawrence, 631

Leshin, S. See Brodie, Leshin, and Brand, xi

Levene, P. A. See Tipson, Christman, and Levene, 609

Levine, Harold. See Carter, Osman, Levine, and Gamm, xiii

Levine, Victor E. The basal metabolic rate of the Eskimo, lix

Lew, W. See Poo, Lew, and Addis, 69

Long, M. Louisa. See Bischoff, Long, and Rupp, viii

Longenecker, Herbert E. Deposition and utilization of fatty acids. I. Fat synthesis from high carbohydrate and high protein diets in fasted rats, 645

—, **Musulin, R. R., and King, C. G.** Urinary excretion of vitamin C, lx

Lonstein, Ida. See Goettsch, Lonstein, and Hutchinson, 9

Looney, Joseph M. The determination of globulin and albumin in blood serum by the photoelectric colorimeter, lx
—. See JELLINEK and LOONEY, 621
Loring, Hubert S. The diffusion constants and approximate molecular weights of tobacco mosaic virus nucleic acid and yeast nucleic acid, lxi
Lowry, Oliver H. Factors affecting the distribution of salts and water in muscle, lxii
Ludewig, Stephan. The effect of partial hepatectomy on the liver lipids, lxii
Lukens, F. D. W. See STADIE, LUKENS, and ZAPP, xcviii
Lyman, Carl M., and **Barron, E. S. Guzman.** Oxidations produced by the kidney in experimental nephritis, lxii
Lyons, Mary. See CORLEY and LYONS, xvii

M

Macy, Icie G. See BEACH, ERICKSON, BERNSTEIN, WILLIAMS, and MACY, 339
Main, Edna R., and **Shinn, Lawrance E.** The determination of hydrogen peroxide in bacterial cultures, 417
Malloy, Helga Tait. See EVELYN and MALLOY, xxvii
Mangun, George, and **Myers, Victor C.** Normal creatine, phosphorus, and potassium content of human cardiac and voluntary muscle, lxiii
—. See MUNTWYLER, MAUTZ, MANGUN, and MELLORS, lxxiv
Marron, T. Urban, and **Moreland, Ferrin B.** Investigations on surface tension and enzyme activity, lxiv
Marsh, Norman. See KOEHLER, MARSH, and HILL, liii
Marshall, E. K., Jr. See BRATTON and MARSHALL, 537
Mason, Morton F. Blood iron studies in the fetal guinea pig, lxiv
Mautz, Frederick R. See MUNTWYLER, MAUTZ, MANGUN, and MELLORS, lxxiv
McCarthy, Earl F., and **Van Slyke, Donald D.** Diurnal variations of hemoglobin in the blood of normal men, 567
McCarthy, James F., Green, Lowell F., and **King, C. G.** The substrate specificity and inhibition characteristics of two copper-protein "oxidases," 455
—. See GREEN, MCCARTHY, and KING, 447
McCay, C. M. See PAUL and MCCAY, lxxvii
McClendon, J. F., and **Rice, Carl O.** Blood hormone iodine and iodide reserve in relation to basal metabolic rate, lxv
McCullagh, D. Roy, and **Daoust, Robert.** Effect of castration on blood androgens, lxv
McDaniel, L. E. See WOOLLEY and MCDANIEL, cxiii
McHenry, E. W., and **Gavin, Gertrude.** The B vitamins and fat metabolism. II. The effect of thiamine upon the synthesis of body fat in pigeons, 45

McHenry, E. W., and Gavin, Gertude. The effect of several B vitamins and of choline upon liver and body fat, lxvi

McJunkin, F. A. See Tweedy, Templeton, Patras, McJunkin, and McNamara, 407

McMeekin, Thomas L. Purification of the choline esterase of serum and the crystallization of serum albumins, lxvi

McNamara, E. W. See Tweedy, Templeton, Patras, McJunkin, and McNamara, 407

Medes, Grace. Enzymic oxidation of sulfur-containing amino acids, lxvii

Mellors, Robert C. See Muntwyler, Mautz, Mangun, and Mellors, lxxiv

Melnick, Daniel, and Field, Henry, Jr. Urinary excretion of thiamine, lxviii

Member, Samuel, and Bruger, Maurice. Blood cholesterol in rabbits during experimental herpes virus infection treated with vitamin B_1 and with sulfanilamide, lxix

Meyer, Curtis E. Endogenous citric acid production, lxix

Meyer, Karl, Smyth, Elizabeth M., and Dawson, Martin H. The isolation of a mucopolysaccharide from synovial fluid, 319, lxx

Miller, Benjamin F., Baker, Zelma, and Allinson, M. J. Carl. A specific, enzymatic method for the analysis of creatine and creatinine in tissues, and its application to studies of the distribution and interrelationship of creatine and creatinine in mammalian tissues, lxx

Mitchell, Helen S., Cook, Gladys M., and O'Brien, Katherine L. Comparison of several calcium salts as to their effect upon lactose utilization, lxxi

Moetsch, J. C. See Raiziss, Severac, Moetsch, and Clemence, lxxxi

Montague, John R. See Todd, Montague, Dittebrandt, and West, ciii

Montgomery, M. L., Entenman, C., and Chaikoff, I. L. The liver lipids of dogs subjected to ligation of the external pancreatic ducts, 387

Moreland, Ferrin B. See Marron and Moreland, lxiv

Morgan, Agnes Fay, Hendricks, Jeanette B., and Shimotori, Nobuko. Hypervitaminoses D_2 and D_3 in rats as affected by calcium and phosphorus of the diet, lxxii

Morgareidge, Kenneth. See O'Brien and Morgareidge, lxxv

Morse, Minerva. The effect of phosphate administration on the capacity of the dog for work, lxxiii

Moyer, A. W. See du Vigneaud, Chandler, Moyer, and Keppel, cviii

Muntwyler, Edward, Mautz, Frederick R., Mangun, George, and Mellors, Robert C. Electrolyte and water exchanges between muscle and blood in adrenal insufficiency, lxxiv

Murrill, William A., and **Newburgh, L. H.** Constancy of the chemical composition of serum proteins regenerated under various dietary régimes, lxxiv

Musulin, R. R. See LONGENECKER, MUSULIN, and KING, lx

Myers, Victor C. See MANGUN and MYERS, lxiii

N

Neurath, Hans, and **Saum, Arthur M.** The denaturation of serum albumin. Diffusion and viscosity measurements of serum albumin in the presence of urea, 347

Newburgh, L. H. See MURRILL and NEWBURGH, lxxiv

Nordbö, R. The concentration of ionized magnesium and calcium in milk, 745

O

O'Brien, Brian, and **Morgareidge, Kenneth.** The effect of phosphorus on the biological estimation of vitamin D activity, lxxv

O'Brien, Katherine L. See MITCHELL, COOK, and O'BRIEN, lxxi

Okey, Ruth, and **Greaves, Vera.** The production of anemia by cholesterol feeding, lxxvi

Oldham, Helen. See SCHLUTZ and OLDHAM, lxxxvi

Oncley, J. L. See FERRY, COHN, ONCLEY, and BLANCHARD, xxviii

Orten, Aline Underhill. See ORTEN and ORTEN, lxxvii

Orten, James M., and **Orten, Aline Underhill.** The relation of dietary protein to hemoglobin formation in the rat, lxxvii

— and **Smith, Arthur H.** On the site of the formation of citric acid in the animal organism, 101

Osman, Elizabeth. See CARTER, OSMAN, LEVINE, and GAMM, xiii

P

Page, Irvine H. See HELMER and PAGE, xlii

Parrott, E. M. See HOGAN and PARROTT, xlvi

Patras, Mary C. See TWEEDY, TEMPLETON, PATRAS, MCJUNKIN, and MCNAMARA, 407

Paul, Henry, and **McCay, C. M.** The influence of the melting points of fats on their utilization by Herbivora, lxxvii

Paul, W. D. See GIBSON, FINDLAYSON, and PAUL, xxxiii

Pearlstein, Leonard. See KASSELL, PEARLSTEIN, BRAND, and CAHILL, p. 1

Perlman, I., and **Chaikoff, I. L.** Radioactive phosphorus as an indicator of phospholipid metabolism. VII. The influence of cholesterol upon phospholipid turnover in the liver, 735

Peterson, V. E. See REINEKE, PETERSON, and TURNER, 1

Peterson, W. H. See SNELL and PETERSON, xciv

Petrovsky, David D. See SOBEL, YUSKA, PETROVSKY, and KRAMER, xcvi

Pett, L. B. The prevalence and importance of hypovitaminosis A, lxxviii

Phillips, Paul H. See ENGEL and PHILLIPS, xxv

Pierce, H. B. See FROESCHLE and PIERCE, xxxi

Pizzolato, Philip. See BEARD, KOVEN, and PIZZOLATO, vii

Poo, L. J., Lew, W., and **Addis, T.** Protein anabolism of organs and tissues during pregnancy and lactation, 69

Power, Marschelle H., Goudsmit, Arnoldus, Jr., and **Keith, Norman M.** Sulfate and sucrose clearances in normal men, lxxix

Pucher, George W. See VICKERY and PUCHER, 685, 703

Pugsley, L. I. The determination of the vitamin A value of tissue of low oil content by the Carr-Price reaction and the percentage of oil on the same sample, lxxx

Q

Quick, Armand J. The concentration of prothrombin in infant blood, lxxx

Quon, S. See DEUEL, HALLMAN, and QUON, xix

R

Raiziss, George W., Severac, M., Moetsch, J. C., and **Clemence, Leroy W.** The antipneumococcic effect of sulfapyridine and related compounds in mice, lxxxi

Randall, Lowell O. The effects of insulin on serum lipids and choline esterase in schizophrenic subjects, lxxxii

Randle, S. B. See KOHLER, RANDLE, and WAGNER, lv

Raney, B. B. See HARGER, BRIDWELL, and RANEY, xxxviii

Ratish, Herman D., Bullowa, Jesse G. M., Ames, James B., and **Scudi, John V.** Urinary excretion products of sulfanilyl-2-aminopyridine, 279

Ratner, S. See SCHOENHEIMER, RATNER, and RITTENBERG, lxxxviii

Reineke, E. P., Peterson, V. E., and **Turner, C. W.** The partition of the serum globulins of the dairy goat, 1

Reiser, Raymond, and **Hanes, Frederick M.** Serum and urinary phosphorus changes in man after olive oil ingestion, lxxxii

Remington, Roe E. See HARRIS and REMINGTON, xl

Rice, Carl O. See McCLENDON and RICE, lxv

Riddle, Oscar. See BATES and RIDDLE, v

Rittenberg, D., Keston, Albert S., and **Graff, Samuel.** Further studies on ammonia metabolism, lxxxiii

—, **Schoenheimer, Rudolf,** and **Keston, Albert S.** Studies in protein metabolism. IX. The utilization of ammonia by normal rats on a stock diet, 603

Rittenberg, D. See SCHOENHEIMER, RATNER, and RITTENBERG, lxxxviii

Roberts, Richard G. Some reactions of ammonolyzed insulin, 597

Roe, Joseph H., and **Hall, James M.** The vitamin C content of human urine and its determination through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid, 329

Rose, Anton R., Schattner, Fred, and **Exton, William G.** The determination of cholesterol, lxxxiv

Ross, A. Frank, and **Stanley, W. M.** The amino acids of tobacco mosaic virus protein, lxxxiv

Routh, Joseph I. The decomposition of cysteine in aqueous solution, lxxxv

Rupp, J. Jerome. See BISCHOFF, LONG, and RUPP, viii

Rusch, H. P. See BAUMANN, JACOBI, and RUSCH, vi

S

Saum, Arthur M. See NEURATH and SAUM, 347

Saunders, Felix. See DORFMAN, HORWITT, KOSER, and SAUNDERS, xx

Schattner, Fred. See ROSE, SCHATTNER, and EXTON, lxxxiv

Schein, Arnold. See BLOCK, 181

Schlutz, Frederic W., and **Oldham, Helen.** The effect of different levels of vitamin B_1 and iron on the retention of iron and the fat content of normal young rats, lxxxvi

Schlutz, Frederic W. See SHUKERS, KNOTT, and SCHLUTZ, xcii

Schmidt, L. H. The therapeutic properties of 2-sulfanilamidopyridine in experimental pneumococcus infections, lxxxvi

Schneider, H., and **Steenbock, H.** A low phosphorus diet and the response of rats to vitamin D_2, 159

— and —. Low phosphorus diets and urinary lithiasis, lxxxvii

Schoenheimer, Rudolf, Ratner, S., and **Rittenberg, D.** Amino acid metabolism with isotopes as indicators, lxxxviii

—. See RITTENBERG, SCHOENHEIMER, and KESTON, 603

Schultz, Fred. See HILL and SCHULTZ, xliv

Schultze, M. O. The effect of deficiencies in copper and iron on oxidation catalysts of rat tissues, lxxxviii

Schwartz, Bernard M. See WINKLER, SMITH, and SCHWARTZ, cxii

Scudi, John V. See RATISH, BULLOWA, AMES, and SCUDI, 279

Sealock, Robert Ridgely, Ziegler, Benjamin, and **Driver, Robert L.** The relation of vitamin C to the metabolism of the melanin pigment precursors, tyrosine and dihydroxyphenylalanine, lxxxix

Sebrell, W. H., and **Daft, Floyd**

Shelton. The effect of cystamine on the albino rat, lxxxix

Seibert, Florence B., and **DuFour, Emma H.** The effect of lyophilizing and other methods of preserving tuberculin protein, xc

Severac, M. See Raiziss, Severac, Moetsch, and Clemence, lxxxi

Sevringhaus, Elmer L. See Heller, Heller, and Sevringhaus, xli

Shepherd, Marion L. See Williams, Shepherd, and Endicott, cxi

Sheppard, Fay, and **Everett, Mark R.** The rôle of cations in carbohydrate oxidation, xci

Shimotori, Nobuko. See Morgan, Hendricks, and Shimotori, lxxii

Shinn, Lawrance E. See Main and Shinn, 417

Shukers, Carroll F., Knott, Elizabeth M., and **Schlutz, Frederic W.** Magnesium balance studies with infants, xcii

Sinclair, Robert Gordon. The rate of turnover of lecithins and cephalins, xcii

Singer, Richard B. See Drabkin and Singer, xxi

Smith, Arthur H. See Orten and Smith, 101

Smith, Henry A., and **Dimick, Mildred K.** Stability of the vitamin B complex to sulfur dioxide and autoclaving, xciii

Smith, Paul K. See Winkler, Smith, and Schwartz, cxii

Smyth, Elizabeth M. See Meyer, Smyth, and Dawson, 319, lxx

Smythe, C. V., and **Harris, T. N.** Some properties of a hemolysin produced by Group A β-hemolytic streptococci, xciv

Snell, E. E., and **Peterson, W. H.** Properties of a new growth factor for lactic acid bacteria, xciv

Sobel, Albert E., and **Sobel, Bernard A.** Microestimation of calcium in one-tenth cubic centimeter of serum, xcv

—, **Yuska, Henry, Petrovsky, David D.,** and **Kramer, Benjamin.** Influence of dietary phosphorus, calcium, and vitamin D in experimental lead poisoning, xcvi

Sobel, Bernard A. See Sobel and Sobel, xcv

Sperry, Warren M., and **Brand, Florence C.** A study of cholesterol esterase in brain and liver, xcvii

Spiegel-Adolf, Mona, and **Henny, George C.** X-ray studies on membranes, xcvii

Sprinson, David B. See Baumann and Sprinson, vi

Stadie, William C., Lukens, F. D. W., and **Zapp, John A., Jr.** The action of insulin upon urea formation and carbohydrate synthesis by liver slices of normal and diabetic animals, xcviii

Stanley, W. M. See Ross and Stanley, lxxxiv

Stare, F. J. See Feinstein and Stare, xxviii

Steenbock, H. See SCHNEIDER and STEENBOCK, 159

Stein, William H. See BERGMANN and STEIN, 217

Steinhardt, Jacinto, and Harris, Milton. The acidic and basic properties of wool protein, xcix

Stekol, Jakob A. Studies on the mercapturic acid synthesis in animals. XI. The detoxication of benzyl chloride, benzyl alcohol, benzaldehyde, and S-benzylhomocysteine in the rabbit and rat, 199

—. XI. On the intermediary metabolism of benzyl chloride in rabbits and rats, c

Stotz, Elmer. See HORECKER, STOTZ, and HOGNESS, 251

Sturges, Stuart. See KNUDSON, STURGES, and BRYAN, 721

Sullivan, Frank A. V., and Cerecedo, Leopold R. The fate of guanylic and adenylic acids in the dog, c

Sullivan, M. X., and Irreverre, Filadelfo. A new and highly specific test for creatinine, ci

—. See HESS and SULLIVAN, 93, xliii

T

Talbot, N. B. See LANGSTROTH and TALBOT, 759

Tannenbaum, Albert. See COHN, TANNENBAUM, THALHIMER, and HASTINGS, 109

Taylor, John Fuller. The hemoglobin-methemoglobin system. The effect of urea on the oxidation-reduction potentials, cii

Templeton, R. D. See TWEEDY, TEMPLETON, PATRAS, MCJUNKIN, and MCNAMARA, 407

Thalhimer, William. See COHN, TANNENBAUM, THALHIMER, and HASTINGS, 109

Thomas, Byron H. See ECK and THOMAS, 257, 267

Thorn, George W., and Howard, R. Palmer. The effect of desoxycorticosterone acetate (synthetic adrenal cortical hormone) on the volume and electrolyte content of the blood plasma in patients with Addison's disease, cii

Tipson, R. Stuart, Christman, Clarence C., and Levene, P. A. The structure of the aldobionic acid from flaxseed mucilage, 609

Todd, W. R., Montague, John R., Dittebrandt, Marlowe, and West, Edward S. Digestion and absorption in a case of regional enteritis with three feet of small intestine, ciii

Toennies, Gerrit, and Callan, Thomas P. Oxidative reactions of methionine, cysteine, and cystine, civ

— and **Kolb, Joseph J.** Methionine studies. II. *dl*-Methionine sulfoxide, 399

Tolksdorf, Sibylle, and Jensen, H. The hormone content of the anterior pituitary of the sperm whale, cv

Treadwell, C. R., and Eckstein, H. C. The composition of the tissue lipids of the Virginia white-tailed deer (*Odocoileus virginianus borealis*), 373

Treadwell, C. R., and **Eckstein, H. C.** Sterol metabolism in the white rat, cv

Trimble, Harry C., and **Keeler, C. E.** The inheritance of high uric acid excretion in dogs, cvi

Turner, C. W. See Reineke, Peterson, and Turner, 1

Tweedy, Wilbur R., Templeton, R. D., Patras, Mary C., McJunkin, F. A., and **McNamara, E. W.** Studies on the effects of calciferol in the thyroparathyroidectomized-nephrectomized rat, 407

V

Van Slyke, Donald D. See McCarthy and Van Slyke, 567

Vennesland, Birgit. The oxidation-reduction potential requirements of a non-spore-forming obligate anaerobe, cvi

Venning, Eleanor Hill, Weil, Paul G., and **Browne, J. S. L.** Excretion of sodium pregnanediol glucuronidate in the adreno-genital syndrome, cvii

Vickery, Hubert Bradford, and **Pucher, George W.** The loss of carbon from excised rhubarb leaves during culture, 685

— and —. The metabolism of amides in green plants. III. The mechanism of amide synthesis, 703

du Vigneaud, Vincent, Chandler, Joseph P., Moyer, A. W., and **Keppel, Dorothy M.** The ability of homocystine plus choline to support growth of the white rat on a methionine-free diet, cviii

du Vigneaud, Vincent. See Kies, Dyer, Wood, and du Vigneaud, 207

Virtue, Robert W., and **Doster-Virtue, Mildred E.** Studies on the production of taurocholic acid in the dog. IV. Cysteine, homocysteine, and thioglycolic acid, 665

W

Wagner, J. R. See Kohler, Randle, and Wagner, lv

Webb, T. J., and **Kniazuk, Michael.** Spectrophotometric examination of blood from animals receiving sulfanilamide, 511

Weil, Paul G. See Venning, Weil, and Browne, cvii

Wendel, Nannette M. See Wendel and Wendel, cix

Wendel, William B., and **Wendel, Nannette M.** Nature of the abnormal pigment in the blood of individuals receiving sulfanilamide treatment, cix

West, Edward S. See Todd, Montague, Dittebrandt, and West, ciii

White, Abraham, and **Bonsnes, Roy W.** Some properties of prolactin, cix

—. See White and White, cx

White, Julius, and **White, Abraham.** Effect of oral administration of methylcholanthrene and of benzpyrene on the growth of the rat, cx

Wilde, W. S. The distribution of potassium in the cat after intravascular injection, 309

Williams, Edwin G. Blood concentration in morphine addicts, cxi
Williams, Harold H., Shepherd, Marion L., and Endicott, Elizabeth E. Fat excretion and the fecal lipid distribution of average, healthy children, cxi
—. See BEACH, ERICKSON, BERNSTEIN, WILLIAMS, and MACY, 339
Williams, Roger J. See EAKIN and WILLIAMS, xxiii
Wilson, D. Wright. See GURIN, BACHMAN, and WILSON, 525, xxxvii
Wilson, J. W. See JOHNSON and WILSON, xlviii
Windsor, Emanuel. See KOEHLER, WINDSOR, and HILL, liv
Winkler, Alexander W., Smith, Paul K., and Schwartz, Bernard M. The distribution in the body of magnesium salts injected intravenously, cxii
Winter, Irwin C. Fat metabolism following liver injury. Decreased iodine number of tissue fatty acids of male rats following carbon tetrachloride administration, 283
Wood, John L. See KIES, DYER, WOOD, and DU VIGNEAUD, 207
Woolley, D. W., and McDaniel, L. E. Concentration of a growth factor for *Clostridium butylicum*, cxiii
Wyckoff, Ralph W. G. The ultracentrifugal analysis of the latent mosaic virus protein, 729

Young, E. Gordon. Ultracentrifugal and electrophoretic analysis of the native proteins in egg white, cxiv
Yuska, Henry. See SOBEL, YUSKA, PETROVSKY, and KRAMER, xcvi

Zapp, John A., Jr. See STADIE, LUKENS, and ZAPP, xcviii
Ziegler, Benjamin. See SEALOCK, ZIEGLER, and DRIVER, lxxxix
Zimmerman, Walter J. See GAEBLER and ZIMMERMAN, xxxii

INDEX TO SUBJECTS

A

Acetone-glyceraldehyde: (Baer and Fischer) 463, 475, 491

Acetone glycerol: *d*(+)-, glycerides, optically active, synthesis (Baer and Fischer) 475

—, preparation (Baer and Fischer) 463

Aconite: Alkaloids (Jacobs, Elderfield, and Craig) 439

Acrodynia: Vitamin B_6 deficiency, relation (Dann) xviii

Addison's disease: Blood plasma volume and electrolytes, desoxycorticosterone acetate effect (Thorn and Howard) cii

Adenosinetriphosphate: Bone phosphatase, hydrolysis (Hitchings and Fuller) xlv

Venom phosphatase, hydrolysis (Hitchings and Fuller) xlv

Adenylic acid: Fate (Sullivan and Cerecedo) c

Adrenal: Cortex hormone. *See also* Desoxycorticosterone acetate

Insufficiency, muscle and blood electrolyte and water exchange (Muntwyler, Mautz, Mangun, and Mellors) lxxiv

Adrenalin: Blood, determination (Bloor) ix

Metabolism effect (Dill, Johnson, and Daly) xx

Adreno-genital syndrome: Sodium pregnanediol glucuronidate excretion (Venning, Weil, and Browne) cvii

Albumin(s): Blood serum crystallization (McMeekin) lxvi

— —, denaturation (Neurath and Saum) 347

— —, determination, colorimetric (Looney) lx

— —, diffusion and viscosity measurements, urea presence (Neurath and Saum) 347

Egg. *See* Egg albumin

Alcohol(s): Body fluids, determination, aerometric (Harger, Bridwell, and Raney) xxxviii

Sugar (Carr and Forman) 425

Water, determination, aerometric (Harger, Bridwell, and Raney) xxxviii

Aldobionic acid: Flaxseed mucilage, structure (Tipson, Christman, and Levene) 609

Alkaloids: Aconite (Jacobs, Elderfield, and Craig) 439

Ergot (Jacobs and Craig) 715

Aluminum: Succinic dehydrogenase-cytochrome system, effect (HORECKER, STOTZ, and HOGNESS) 251
Amides: Plant, green, metabolism (VICKERY and PUCHER) 703
—, —, synthesis mechanism (VICKERY and PUCHER) 703
Amidopyridine: 2-Sulfanil-, pneumococcus infection, effect (SCHMIDT) lxxxvi
Amino acid(s): Anemia, deaminized casein-produced, effect (GUERRANT and HOGAN) 363
Blood cell, red, posthemolytic residue (BEACH, ERICKSON, BERNSTEIN, WILLIAMS, and MACY) 339
Determination (BERGMANN and STEIN) 217
Eukeratins (BLOCK) 181
Hair (BLOCK) 181
Horn (BLOCK) 181
Hydroxy group, property (HAMMETT) xxxvii
Metabolism, isotopes as indicators (SCHOENHEIMER, RATNER, and RITTENBERG) lxxxviii
Multivalent (GREENSTEIN) 241
Oxidation, kidney and liver, thyroid feeding, and thyroidectomy effect (KLEIN) 659
Racemization, acetylation with ketene (CAHILL) xii
Sulfur-containing, enzyme oxidation (MEDES) lxvii
Amino acid(s)—*continued:*
Tobacco mosaic virus protein (ROSS and STANLEY) lxxxiv
Wool (BLOCK) 181
Aminopyridine: Sulfanilyl-2-, urine excretion products (RATISH, BULLOWA, AMES, and SCUDI) 279
Ammonia: Metabolism (RITTENBERG, KESTON, and GRAFF) lxxxiii
Utilization (RITTENBERG, SCHOENHEIMER, and KESTON) 603
Ammonium carbonate: Glycine from, carbonate effect (DUNN, BUTLER, and FRIEDEN) xxii
Ammonium hydroxide: Glycine from, carbonate effect (DUNN, BUTLER, and FRIEDEN) xxii
Anaerobe: Non-spore-forming, oxidation-reduction potential requirements (VENNESLAND) cvi
Androgenic substances: Blood, castration effect (McCULLAGH and DAOUST) lxv
Androsterone: *m*-Dinitrobenzene reaction compounds, absorption spectra (LANGSTROTH and TALBOT) 759
Anemia: Chick, vitamin deficiency, relation (HOGAN and PARROTT) xlvi
Cholesterol production (OKEY and GREAVES) lxxvi
Deaminized casein-produced, amino acid effect (GUERRANT and HOGAN) 363

Anesthesia: Barbituric acids, ionization, and anesthetic bases, relation (CLOWES, KELTCH, and KRAHL) xv

Antuitrin: -T, creatine-creatinine metabolism, relation (KOVEN and BEARD) lvi

Apparatus: Manometer, oil, Van Slyke-Neill gas apparatus (HANKE) xxxviii

Ascorbic acid: Vitamin A utilization, relation (KIMBLE and GORDON) lii

Aspartylhistidine: (GREENSTEIN and KLEMPERER) 245

Autolysis: Cathepsin, effect (EDER, BRADLEY, and BELFER) 551

B

Bacteria: Lactic acid, growth factor, properties (SNELL and PETERSON) xciv

See also Anaerobe, *Clostridium*

Bacterial cultures: Hydrogen peroxide determination (MAIN and SHINN) 417

Barbituric acids: Ionization, anesthetic action, relation (CLOWES, KELTCH, and KRAHL) xv

Substituted, dissociation constants, apparent (KRAHL) lvii

Basal metabolism: Blood thyroid hormone, iodine and iodide, relation (McCLENDON and RICE) lxv

Eskimo (LEVINE) lix

Bases: Anesthetic, ionization, effect (CLOWES, KELTCH, and KRAHL) xv

Bean: Soy, proteins, storage effect (JONES and GERSDORFF) xlix

Benzaldehyde: Detoxication, animal body (STEKOL) 199

Benzedrine: Metabolism effect (DILL, JOHNSON, and DALY) xx

Benzpyrene: Growth effect (WHITE and WHITE) cx

Benzyl alcohol: Detoxication, animal body (STEKOL) 199

Benzyl chloride: Detoxication, animal body (STEKOL) 199

Metabolism, intermediary (STEKOL) c

Benzylhomocysteine: S-, detoxication, animal body (STEKOL) 199

Biochemical variables: Normal men (JELLINEK and LOONEY) 621

Biotic acid: Liver, growth substance, new (EAKIN and WILLIAMS) xxiii

Blood: Adrenalin determination (BLOOR) ix

Androgenic substances, castration effect (McCULLAGH and DAOUST) lxv

Carbon dioxide effect (COHN, TANNENBAUM, THALHIMER, and HASTINGS) 109

Chicken, urea determination (HOWELL) 573

Cholesterol, herpes, vitamin B_1 and sulfanilamide effect (MEMBER and BRUGER) lxix

—, ultraviolet irradiation effect (KNUDSON, STURGES, and BRYAN) 721

Blood—*continued:*
  Concentration, morphine addicts (Williams) cxi
  Electrolytes, muscle and, exchange, adrenal insufficiency (Muntwyler, Mautz, Mangun, and Mellors) lxxiv
  Hemoglobin, diurnal variations (McCarthy and Van Slyke) 567
  Infant, prothrombin (Quick) lxxx
  Iron, fetus (Mason) lxiv
  Maltose determination, glucose presence (Koehler, Marsh, and Hill) liii
  Oxygen effect (Cohn, Tannenbaum, Thalhimer, and Hastings) 109
  Physicochemical system, lactic acid in (Johnson and Wilson) xlviii
  Pigment, abnormal, sulfanilamide relation (Wendel and Wendel) cix
  Pneumococcus Type XIV polysaccharide, capsular, specific substance, relation (Goebel, Beeson, and Hoagland) xxxiv
  Pneumonia, oxygen and carbon dioxide effect (Cohn, Tannenbaum, Thalhimer, and Hastings) 109
  Quinine determination (Kyker and Andrews) lviii
  Sulfanilamide effect (Webb and Kniazuk) 511
  Thyroid hormone iodine and iodide, basal metabolism, relation (McClendon and Rice) lxv

Blood—*continued:*
  Water, muscle and, exchange, adrenal insufficiency (Muntwyler, Mautz, Mangun, and Mellors) lxxiv
Blood cell: Red, absorption spectra, light scattering effect (Drabkin and Singer) xxi
  —, posthemolytic residue (Hoffman and Beach) xlv
  —, — —, amino acids (Beach, Erickson, Bernstein, Williams, and Macy) 339
  —, stroma, polycythemia (Hoffman and Beach) xlv
Blood plasma: Electrolytes, Addison's disease, desoxycorticosterone acetate effect (Thorn and Howard) cii
  Volume, Addison's disease, desoxycorticosterone acetate effect (Thorn and Howard) cii
Blood serum: Albumin, denaturation (Neurath and Saum) 347
  — determination, colorimetric (Looney) lx
  —, diffusion and viscosity measurements, urea presence (Neurath and Saum) 347
  Albumins, crystallization (McMeekin) lxvi
  Calcium, determination, micro- (Sobel and Sobel) xcv
  Choline esterase, purification (McMeekin) lxvi
  — —, schizophrenia, insulin effect (Randall) lxxxii
  Globulin determination, colorimetric (Looney) lx
  Globulins, partition (Reineke, Peterson, and Turner) 1

Blood serum—*continued:*
  Lipids, schizophrenia, insulin effect (Randall) lxxxii
  Phosphorus, olive oil ingestion, effect (Reiser and Hanes) lxxxii
  Proteins, mental disease (Kondritzer) lv
  —, regenerated, diet effect (Murrill and Newburgh) lxxiv
Body fluids: Alcohol determination, aerometric (Harger, Bridwell, and Raney) xxxviii
Bone: Phosphatase, adenosinetriphosphate hydrolysis (Hitchings and Fuller) xlv
  —, temperature effect (Bodansky) x
Brain: Cholesterol esterase (Sperry and Brand) xcvii
Bromide: Extracellular fluid measurement, use (Brodie, Leshin, and Brand) xi

C

Calciferol: Thyroparathyroidectomy-nephrectomy, effect (Tweedy, Templeton, Patras, McJunkin, and McNamara) 407
Calcium: Blood serum, determination, micro- (Sobel and Sobel) xcv
  Dietary, hypervitaminoses D_2 and D_3, effect (Morgan, Hendricks, and Shimotori) lxxii
  —, lead poisoning, influence (Sobel, Yuska, Petrovsky, and Kramer) xcvi
Calcium—*continued:*
  Ionized, milk (Nordbö) 745
Calcium phosphates: Solubility (Hodge) xlv
Calcium salts: Lactose utilization, comparison (Mitchell, Cook, and O'Brien) lxxi
Calculi: Urine, phosphorus-low diet, relation (Schneider and Steenbock) lxxxvii
Carbohydrate: Dietary, uric acid excretion, effect (Adlersberg and Ellenberg) 379
  Gonadotropic hormone, urine, pregnancy (Gurin, Bachman, and Wilson) xxxvii
  -High diets, fat synthesis, fasting (Longenecker) 645
  Muscle, working (Flock and Bollman) xxx
  Oxidation, cations, rôle (Sheppard and Everett) xci
  Synthesis, liver, normal and diabetic, insulin action (Stadie, Lukens, and Zapp) xcviii
Carbon: Rhubarb leaves, excised, loss during culture (Vickery and Pucher) 685
Carbonate: Glycine synthesis from chloroacetic acid, ammonium hydroxide, and ammonium carbonate, effect (Dunn, Butler, and Frieden) xxii
Carbon dioxide: Blood, influence (Cohn, Tannenbaum, Thalhimer, and Hastings) 109
Carbon tetrachloride: Tissue fatty acids, administration effect (Winter) 283

Carcinoma: Mammary, phospholipid metabolism, radioactive phosphorus as indicator (Jones, Chaikoff, and Lawrence) 631
Cardiac: *See* Heart
Carotene: Goiter from low iodine intake, effect (Harris and Remington) xl
Casein: Deaminized, anemia from, amino acid effect (Guerrant and Hogan) 363
Castration. Blood androgenic substances, effect (McCullagh and Daoust) lxv
Cathepsin: Autolysis effect (Eder, Bradley, and Belfer) 551
  Survival (Eder, Bradley, and Belfer) xxiii
Cations: Carbohydrate oxidation, rôle (Sheppard and Everett) xci
Cephalin(s): Formation rate, body (Chargaff) xiv
  — —, —, unstable isotopes as indicator (Chargaff) 587
  Turnover (Sinclair) xcii
Cereal: Starch, stomach emptying rate, effect (Froeschle and Pierce) xxxi
Children: Fat excretion (Williams, Shepherd, and Endicott) cxi
  Fecal lipid distribution (Williams, Shepherd, and Endicott) cxi
Chlorella pyrenoidosa: Respiration and photosynthesis inhibition (Green, McCarthy, and King) 447
Chloroacetic acid: Glycine from, carbonate effect (Dunn, Butler, and Frieden) xxii
Cholanthrene: Methyl-, growth effect (White and White) cx
Cholesterol: Anemia from (Okey and Greaves) lxxvi
  Blood, herpes, vitamin B_1 and sulfanilamide effect (Member and Bruger) lxix
  —, ultraviolet irradiation effect (Knudson, Sturges, and Bryan) 721
  Determination (Rose, Schattner, and Exton) lxxxiv
  Esterase, brain and liver (Sperry and Brand) xcvii
  Liver, phospholipid metabolism influence, radioactive phosphorus as indicator (Perlman and Chaikoff) 735
  Skin, ultraviolet irradiation effect (Knudson, Sturges, and Bryan) 721
  Tumor tissue, ultraviolet irradiation effect (Knudson, Sturges, and Bryan) 721
Choline: Body fat, effect (McHenry and Gavin) lxvi
  Esterase, blood serum, purification (McMeekin) lxvi
  —, schizophrenia, insulin effect (Randall) lxxxii
  —, specificity (Glick) xxxiv
  Homocystine and, methionine-free diet, growth effect (du Vigneaud, Chandler, Moyer, and Keppel) cviii

Choline—*continued:*
Liver fat, effect (McHenry and Gavin) lxvi
Chromium: Succinic dehydrogenase-cytochrome system, effect (Horecker, Stotz, and Hogness) 251
Cinchona: Series, chemical constitution and antipneumococcus activity (Cretcher) xviii
Citric acid: Endogenous, production (Meyer) lxix
Formation site, animal organism (Orten and Smith) 101
Clostridium butylicum: Growth factor (Woolley and McDaniel) cxiii
Cobalt: Hemoglobin production, milk diet, rôle (Frost and Elvehjem) xxxi
Collagen: Amino acids, determination (Bergmann and Stein) 217
Copper: Catalysis inhibitors, respiration and photosynthesis, *Chlorella pyrenoidosa*, effect (Green, McCarthy, and King) 447
Hemoglobin production, milk diet, rôle (Frost and Elvehjem) xxxi
-Protein oxidases, properties (McCarthy, Green, and King) 455
Tissue oxidation, effect (Schultze) lxxxviii
Cortin: -Like compounds, activity (Kendall) li
Creatine: Creatinine and, relation (Beard, Koven, and Pizzolato) vii
Creatine—*continued:*
-Creatinine metabolism, hormones, relation (Koven and Beard) lvi
— —, progestin and antuitrin-T effect (Koven and Beard) lvi
Muscle, cardiac and voluntary (Mangun and Myers) lxiii
Tissues, creatinine and, distribution and interrelationship (Miller, Baker, and Allinson) lxx
—, determination, enzymatic (Miller, Baker, and Allinson) lxx
Creatinine: Creatine and, relation (Beard, Koven, and Pizzolato) vii
Creatine-, metabolism, hormones, relation (Koven and Beard) lvi
—, —, progestin and antuitrin-T effect (Koven and Beard) lvi
Test (Sullivan and Irreverre) ci
Tissues, creatine and, distribution and interrelationship (Miller, Baker, and Allinson) lxx
—, determination, enzymatic (Miller, Baker, and Allinson) lxx
Crop sac: Response, prolactin, sex difference (Bates and Riddle) v
Cystamine: (Sebrell and Daft) lxxxix
Cysteine: S-Benzylhomo-, detoxication, animal body (Stekol) 199

Cysteine—*continued:*
Decomposition (Routh) lxxxv
Homo-, taurocholic acid production, effect (Virtue and Doster-Virtue) 665
Oxidation (Toennies and Callan) civ
Taurocholic acid production, effect (Virtue and Doster-Virtue) 665

Cystine: Diglycyl-*l*-, synthesis (Greenstein) 241
N,N′-Dimethyl-, optical isomers, utilization (Kies, Dyer, Wood, and du Vigneaud) 207
Oxidation (Toennies and Callan) civ
Proteins, deaminized (Hess and Sullivan) 93

Cystine anhydrides: Acid sensitivity (Hess and Sullivan) xliii

Cystine peptides: Acid sensitivity (Hess and Sullivan) xliii

Cystinuria: Diuresis effect (Andrews and Andrews) iii
Sulfanilamide metabolism (Kassell, Pearlstein, Brand, and Cahill) p. l

Cytochrome: Succinic dehydrogenase-, system, effect (Horecker, Stotz, and Hogness) 251

Deer: Virginia white-tailed, tissue lipids, composition (Treadwell and Eckstein) 373

Dehydrogenase: Succinic, -cytochrome system, effect (Horecker, Stotz, and Hogness) 251

Delphinine: (Jacobs and Craig) 431
Oxo- (Jacobs and Craig) 431

Desoxycorticosterone acetate: Blood plasma volume and electrolytes, Addison's disease, effect (Thorn and Howard) cii

Diabetes: Liver urea formation and carbohydrate synthesis, insulin action (Stadie, Lukens, and Zapp) xcviii
Maltose utilization (Koehler, Windsor, and Hill) liv
Phlorhizin, pituitary, anterior, growth preparation, metabolism effect (Gaebler and Zimmerman) xxxii

Diet: Blood serum proteins, regenerated, effect (Murrill and Newburgh) lxxiv
Calcium, hypervitaminoses D_2 and D_3, effect (Morgan, Hendricks, and Shimotori) lxxii
—, lead poisoning, influence (Sobel, Yuska, Petrovsky, and Kramer) xcvi
Carbohydrate-high, fat synthesis, fasting (Longenecker) 645
Carbohydrate, uric acid excretion, effect (Adlersberg and Ellenberg) 379
Fat, uric acid excretion, effect (Adlersberg and Ellenberg) 379
Methionine-free, homocystine plus choline, growth effect

Diet—*continued:*
(du Vigneaud, Chandler, Moyer, and Keppel) cviii
Phosphorus, hypervitaminoses D_2 and D_3, effect (Morgan, Hendricks, and Shimotori) lxxii
—, lead poisoning, influence (Sobel, Yuska, Petrovsky, and Kramer) xcvi
Phosphorus-low, urinary calculi, relation (Schneider and Steenbock) lxxxvii
—, vitamin D_2 effect (Schneider and Steenbock) 159
Protein, growth, liver fat and, relation (Griffith) xxxvi
—, hemoglobin formation, relation (Orten and Orten) lxxvii
Protein-high, fat synthesis, fasting (Longenecker) 645
Thiamine requirement (Arnold) iv
Tumor effect (Baumann, Jacobi, and Rusch) vi
Vitamin D, lead poisoning, influence (Sobel, Yuska, Petrovsky, and Kramer) xcvi
Diglycyl-*l*-cystine: Synthesis (Greenstein) 241
Dihydrolysergic acid: Dimethylindole from (Jacobs and Craig) 715
Dihydroxyphenylalanine: Metabolism, vitamin C relation (Sealock, Ziegler, and Driver) lxxxix
Dimethylcystine: N,N′-, optical isomers, utilization (Kies, Dyer, Wood, and du Vigneaud) 207
Dimethylindole: Dihydrolysergic acid derivative (Jacobs and Craig) 715
Dipeptides: Racemization, acetylation with ketene (Cahill) xii
Dysentery: Organism, nicotinic acid determination, use (Dorfman, Horwitt, Koser, and Saunders) xx

E

Earths: Rare, succinic dehydrogenase-cytochrome system, effect (Horecker, Stotz, and Hogness) 251
Edestin: Solutions, sulfhydryl groups (Greenstein) 233
Egg: White, proteins, ultracentrifugal and electrophoretic analysis (Young) cxiv
Egg albumin: Denaturation, ultraviolet radiant energy, effect (Bernhart) 289
Molecular splitting, ultraviolet radiant energy, effect (Bernhart) 289
Electrolytes: Blood plasma, Addison's disease, desoxycorticosterone acetate effect (Thorn and Howard) cii
Kidney (Eichelberger and Bibler) xxiv
Muscle and blood, exchange, adrenal insufficiency (Muntwyler, Mautz, Mangun, and Mellors) lxxiv
Endocrine factors: Tumors, effect (Bischoff, Long, and Rupp) viii

Enteritis: Digestion and absorption (Todd, Montague, Dittebrandt, and West) ciii

Enzyme(s): Activity, surface tension, relation (Marron and Moreland) lxiv
  Oxidation, amino acids, sulfur-containing (Medes) lxvii
  Tissue creatine, determination (Miller, Baker, and Allinson) lxx
  — creatinine, determination (Miller, Baker, and Allinson) lxx
  See also Dehydrogenase, Esterase, Oxidase, Phosphatase

Ergot: Alkaloids (Jacobs and Craig) 715

Eskimo: Basal metabolism (Levine) lix

Esterase: Cholesterol, brain and liver (Sperry and Brand) xcvii
  Choline, blood serum, purification (McMeekin) lxvi
  —, schizophrenia, insulin effect (Randall) lxxxii
  —, specificity (Glick) xxxiv

Estrogenic substances: Conjugated, urine, pregnancy, hydrolysis (Heard and Edson) xli

Eukeratins: Amino acids (Block) 181

Excelsin: Solutions, sulfhydryl groups (Greenstein) 233

Exercise: *See also* Muscle, Work

Extracellular fluids: Measurement, bromide use (Brodie, Leshin, and Brand) xi

F

Factor W: Vitamin B complex, relation (Frost and Elvehjem) 23

Fasting: Fat synthesis, high carbohydrate-high protein diets (Longenecker) 645

Fat(s): Absorption, study technique (Deuel, Hallman, and Quon) xix
  Body, iron effect (Schlutz and Oldham) lxxxvi
  —, synthesis, thiamine effect (McHenry and Gavin) 45
  —, vitamin B and choline effect (McHenry and Gavin) lxvi
  —, — B_1 effect (Schlutz and Oldham) lxxxvi
  Dietary, uric acid excretion, effect (Adlersberg and Ellenberg) 379
  Excretion, children (Williams, Shepherd, and Endicott) cxi
  Liver, dietary protein, and growth, relation (Griffith) xxxvi
  —, vitamin B and choline effect (McHenry and Gavin) lxvi
  Metabolism, liver injury, effect (Winter) 283
  —, vitamin B, relation (McHenry and Gavin) 45
  Synthesis, high carbohydrate-high protein diets, fasting (Longenecker) 645
  Utilization, melting point influence (Paul and McCay) lxxvii

Fatty acids: Alkyl derivatives, glycogen formation, relation (Corley and Lyons) xvii
Amino derivatives, glycogen formation, relation (Corley and Lyons) xvii
Deposition and utilization (Longenecker) 645
Metabolism (Carter, Osman, Levine, and Gamm) xiii
Tissue, carbon tetrachloride administration, effect (Winter) 283
Feces: Lipid distribution, children (Williams, Shepherd, and Endicott) cxi
Fetus: Blood iron (Mason) lxiv
Filtrate factor: (Jukes) 35
Fish liver oils: Vitamin A, cyclized (Embree) 187
— —, nature (Hickman) xliii
Flavin: Ribo-, vitamin A utilization, relation (Kimble and Gordon) lii
Flaxseed: Mucilage, aldobionic acid structure (Tipson, Christman, and Levene) 609

G

Gastric: *See* Stomach
Gelatin: Amino acids, determination (Bergmann and Stein) 217
Globin: Solutions, sulfhydryl groups (Greenstein) 233
Globulin(s): Blood serum, determination, colorimetric (Looney) lx
— —, partition (Reineke, Peterson, and Turner) 1
Globulin(s)—*continued:*
Lacto-, dielectric properties (Ferry, Cohn, Oncley, and Blanchard) xxviii
Glucose: Blood maltose determination, effect (Koehler, Marsh, and Hill) liii
Kidney threshold, pregnancy (Gibson, Findlayson, and Paul) xxxiii
Protein conversion, pancreatectomy and phlorhizin effect (Gray, Ivy, and Cuthbert) 173
Tissue maltose determination, effect (Koehler, Marsh, and Hill) liii
Urine maltose determination, effect (Koehler, Marsh, and Hill) liii
Glyceraldehyde: Acetone- (Baer and Fischer) 463, 475, 491
Glycerides: *d*(+)-Acetone glycerol, optically active, synthesis (Baer and Fischer) 475
Glycerol: *d*(+)-Acetone, glycerides, optically active, synthesis (Baer and Fischer) 475
—, preparation (Baer and Fischer) 463
Glycerophosphoric acid: *l*(−)-*α*-, biological, synthesis (Baer and Fischer) 491
Glycine: Ammonium carbonate synthesis, carbonate effect (Dunn, Butler, and Frieden) xxii
— hydroxide synthesis, carbonate effect (Dunn, Butler, and Frieden) xxii

Glycine—*continued:*
  Chloroacetic acid synthesis, carbonate effect (DUNN, BUTLER, and FRIEDEN) xxii
Glycogen: Formation, fatty acids, amino and alkyl derivatives, relation (CORLEY and LYONS) xvii
  Liver, potassium and phosphate relation (FENN) 297
Glycolic acid: Thio-, taurocholic acid production, effect (VIRTUE and DOSTER-VIRTUE) 665
Goiter: Low iodine-induced, vitamin A and carotene effect (HARRIS and REMINGTON) xl
Gonadotropic activity: Augmentation (EVANS, CEITHAML, HINES, and KOCH) xxvi
Gonadotropic hormone: Carbohydrate, urine, pregnancy (GURIN, BACHMAN, and WILSON) xxxvii
  Urine, male, assay (HELLER, HELLER, and SEVRINGHAUS) xli
  —, pregnancy (GURIN, BACHMAN, and WILSON) 525
  —, —, preparation (GURIN, BACHMAN, and WILSON) 525
Grass juice factor: (KOHLER, RANDLE, and WAGNER) lv
Growth: Benzpyrene effect (WHITE and WHITE) cx
  Factor, *Clostridium butylicum* (WOOLLEY and McDANIEL) cxiii
  —, lactic acid bacteria, properties (SNELL and PETERSON) xciv
Growth—*continued:*
  Homocystine plus choline, methionine-free diet, effect (DU VIGNEAUD, CHANDLER, MOYER, and KEPPEL) cviii
  Methylcholanthrene effect (WHITE and WHITE) cx
  Preparation, pituitary, anterior, phlorhizin diabetes, metabolism effect (GAEBLER and ZIMMERMAN) xxxii
  Protein, dietary, liver fat and, relation (GRIFFITH) xxxvi
  Substance, liver, new (EAKIN and WILLIAMS) xxiii
Guanylic acid: Fate (SULLIVAN and CERECEDO) c

H

Hair: Amino acids (BLOCK) 181
Heart: Output, roentgenkymography (KEYS and FRIEDELL) li
  See also Muscle
Hemoglobin: Blood, diurnal variations (McCARTHY and VAN SLYKE) 567
  Formation, dietary protein, relation (ORTEN and ORTEN) lxxvii
  -Methemoglobin oxidation-reduction potentials, urea effect (TAYLOR) cii
  Production, milk diet, iron, copper, and cobalt rôle (FROST and ELVEHJEM) xxxi
  Synthesis, pyrrole-containing pigments, relation (KOHLER, ELVEHJEM, and HART) 501
  See also Methemoglobin

Hemolysin: *β-Streptococcus hæmolyticus*, properties (Smythe and Harris) xciv

Hepatectomy: Liver lipids, effect (Ludewig) lxii

Herpes: Blood cholesterol, vitamin B_1 and sulfanilamide effect (Member and Bruger) lxix

Hippuric acid: Synthesis, biological, *in vitro* (Borsook and Dubnoff) cxiv

Histidine: Aspartyl- (Greenstein and Klemperer) 245

Homocysteine: Taurocholic acid production, effect (Virtue and Doster-Virtue) 665

Homocystine: Choline and, methionine-free diet, growth effect (du Vigneaud, Chandler, Moyer, and Keppel) cviii

Horn: Amino acids (Block) 181

Hydrogen ion concentration: Determination, colorimetric (Evelyn and Malloy) xxvii

Hydrogen peroxide: Bacterial cultures, determination (Main and Shinn) 417

Hypervitaminosis: D_2 and D_3, dietary calcium and phosphorus, effect (Morgan, Hendricks, and Shimotori) lxxii

Hypovitaminosis: A (Pett) lxxviii

I

Indole: Dimethyl-, dihydrolysergic acid derivative (Jacobs and Craig) 715

Infant: Blood, prothrombin (Quick) lxxx

Magnesium balance (Shukers, Knott, and Schlutz) xcii

Insulin: Ammonolyzed, reactions (Roberts) 597

Attenuation, adsorption, interfacial (Johlin) xlviii

Blood serum choline esterase, schizophrenia, effect (Randall) lxxxii

— — lipids, schizophrenia, effect (Randall) lxxxii

Liver carbohydrate synthesis, normal and diabetic, action (Stadie, Lukens, and Zapp) xcviii

— urea formation, normal and diabetic, action (Stadie, Lukens, and Zapp) xcviii

Intestine: Small, digestion and absorption, enteritis (Todd, Montague, Dittebrandt, and West) ciii

See also Enteritis

Iodide: Blood thyroid hormone, basal metabolism, relation (McClendon and Rice) lxv

Iodine: Blood thyroid hormone, basal metabolism, relation (McClendon and Rice) lxv

Low, goiter, vitamin A and carotene effect (Harris and Remington) xl

Ions: Dipolar, lactoglobulin interaction (Ferry, Cohn, Oncley, and Blanchard) xxviii

Lactoglobulin interaction (Ferry, Cohn, Oncley, and Blanchard) xxviii

Iron: Blood, fetus (Mason) lxiv
  Body fat, effect (Schlutz and Oldham) lxxxvi
  Hemoglobin production, milk diet, rôle (Frost and Elvehjem) xxxi
  Iron retention, effect (Schlutz and Oldham) lxxxvi
  Tissue oxidation, effect (Schultze) lxxxviii
Isotopes: Amino acid metabolism, indicators (Schoenheimer, Ratner, and Rittenberg) lxxxviii
  Radioactive, mineral metabolism indicator (Joseph, Cohn, and Greenberg) 673
    (Cohn and Greenberg) xvi
  —, organic material, determination (Chargaff) 579, xiii
  Unstable (Chargaff) 579, 587
  —, lecithin and cephalin formation rate, indicator (Chargaff) 587

K

Keratins: Amino acids (Block) 181
  Eu-, amino acids (Block) 181
Ketene: Amino acid racemization, acetylation with (Cahill) xii
  Dipeptide racemization, acetylation with (Cahill) xii
Kidney: Amino acid oxidation, thyroid feeding and thyroidectomy effect (Klein) 659
Kidney—*continued:*
  Electrolytes (Eichelberger and Bibler) xxiv
  Impairment, muscle water, influence (Eichelberger) 137
  Oxidations, nephritis (Lyman and Barron) lxii
  Proteins (Ferry, Ferry, and Connolly) xxix
  Threshold, glucose, pregnancy (Gibson, Findlayson, and Paul) xxxiii
  Water (Eichelberger and Bibler) xxiv
  See also Nephrectomy, Nephritis

L

Lactation: Organ and tissue protein, metabolism (Poo, Lew, and Addis) 69
Lactic acid: Bacteria, growth factor, properties (Snell and Peterson) xciv
  Blood physicochemical system (Johnson and Wilson) xlviii
Lactoglobulin: Dielectric properties (Ferry, Cohn, Oncley, and Blanchard) xxviii
Lactose: Utilization, calcium salts, comparison (Mitchell, Cook, and O'Brien) lxxi
Lead: Poisoning, dietary phosphorus, calcium, and vitamin D, influence (Sobel, Yuska, Petrovsky, and Kramer) xcvi
Lecithin(s): Formation rate, body (Chargaff) xiv

- **Lecithin(s)**—*continued:*
  - Formation rate, body, unstable isotopes as indicator (Chargaff) 587
  - Turnover (Sinclair) xcii
- **Light:** Scattering, red blood cell absorption spectra, effect (Drabkin and Singer) xxi
- **Lipids:** Blood serum, schizophrenia, insulin effect (Randall) lxxxii
  - Deer, Virginia white-tailed, composition (Treadwell and Eckstein) 373
  - Feces, distribution, children (Williams, Shepherd, and Endicott) cxi
  - Liver, hepatectomy effect (Ludewig) lxii
  - —, pancreatic duct ligation, effect (Montgomery, Entenman, and Chaikoff) 387
- **Liver:** Amino acid oxidation, thyroid feeding and thyroidectomy effect (Klein) 659
  - Biotic acid, growth substance (Eakin and Williams) xxiii
  - Carbohydrate synthesis, normal and diabetic, insulin action (Stadie, Lukens, and Zapp) xcviii
  - Cell tumors, vitamin A relation (Goerner and Goerner) 559
  - Cholesterol esterase (Sperry and Brand) xcvii
  - Fat, choline effect (McHenry and Gavin) lxvi
- **Liver**—*continued:*
  - Fat, dietary protein, and growth, relation (Griffith) xxxvi
  - —, vitamin B effect (McHenry and Gavin) lxvi
  - Fatty, phospholipids (Flock, Hester, and Bollman) 153
  - —, thiamine effect (Engel and Phillips) xxv
  - Glycogen, potassium, and phosphate, relation (Fenn) 297
  - Growth substance, new (Eakin and Williams) xxiii
  - Injury, fat metabolism, effect (Winter) 283
  - Lipids, hepatectomy effect (Ludewig) lxii
  - —, pancreatic duct ligation, effect (Montgomery, Entenman, and Chaikoff) 387
  - Oxidations, manganese and other metals, action (Bernheim and Bernheim) 79
  - Phosphate, glycogen, and potassium, relation (Fenn) 297
  - Phospholipid metabolism, cholesterol influence, radioactive phosphorus as indicator (Perlman and Chaikoff) 735
  - Potassium, phosphate, and glycogen, relation (Fenn) 297
  - Urea formation, normal and diabetic, insulin action (Stadie, Lukens, and Zapp) xcviii
  - *See also* Hepatectomy

Lymphoma: Mammary, phospholipid metabolism, radioactive phosphorus as indicator (JONES, CHAIKOFF, and LAWRENCE) 631
Lymphosarcoma: Mammary, phospholipid metabolism, radioactive phosphorus as indicator (JONES, CHAIKOFF, and LAWRENCE) 631
Lysergic acid: Dihydro-, dimethylindole from (JACOBS and CRAIG) 715

M

Magnesium: Balance, infants (SHUKERS, KNOTT, and SCHLUTZ) xcii
Ionized, milk (NORDBÖ) 745
Magnesium salts: Body distribution, intravenous injection effect (WINKLER, SMITH, and SCHWARTZ) cxii
Maltose: Blood, determination, glucose presence (KOEHLER, MARSH, and HILL) liii
Tissue, determination, glucose presence (KOEHLER, MARSH, and HILL) liii
Urine, determination, glucose presence (KOEHLER, MARSH, and HILL) liii
Utilization, diabetes (KOEHLER, WINDSOR, and HILL) liv
Manganese: Liver oxidations, action (BERNHEIM and BERNHEIM) 79
Manometer: Oil, Van Slyke-Neill gas apparatus (HANKE) xxxviii
Melanin: Pigment precursors, metabolism, vitamin C relation (SEALOCK, ZIEGLER, and DRIVER) lxxxix
Membranes: Roentgen ray diffraction (SPIEGEL-ADOLF and HENNY) xcvii
Mental disease: Blood serum proteins (KONDRITZER) lv
Mercapturic acid: Synthesis, animals (STEKOL) 199, c
Metabolism: Adrenalin effect (DILL, JOHNSON, and DALY) xx
Amides, plants, green (VICKERY and PUCHER) 703
Amino acid, isotopes as indicators (SCHOENHEIMER, RATNER, and RITTENBERG) lxxxviii
Ammonia (RITTENBERG, KESTON, and GRAFF) lxxxiii
Benzedrine effect (DILL, JOHNSON, and DALY) xx
Creatine-creatinine, hormones, relation (KOVEN and BEARD) lvi
—, progestin and antuitrin-T effect (KOVEN and BEARD) lvi
Diabetes, phlorhizin, pituitary, anterior, growth preparation, effect (GAEBLER and ZIMMERMAN) xxxii
Dihydroxyphenylalanine, vitamin C relation (SEALOCK, ZIEGLER, and DRIVER) lxxxix
Fat, liver injury, effect (WINTER) 283
—, vitamin B relation (McHENRY and GAVIN) 45

Metabolism—*continued:*
Fatty acids (Carter, Osman, Levine, and Gamm) xiii
Intermediary, benzyl chloride (Stekol) c
Melanin pigment precursors, vitamin C relation (Sealock, Ziegler, and Driver) lxxxix
Mineral, radioactive isotopes in study (Joseph, Cohn, and Greenberg) 673
(Cohn and Greenberg) xvi
Organ protein, pregnancy and lactation (Poo, Lew, and Addis) 69
Phospholipid, neoplastic tissues, radioactive phosphorus as indicator (Jones, Chaikoff, and Lawrence) 631
—, radioactive phosphorus as indicator (Jones, Chaikoff, and Lawrence) 631
(Perlman and Chaikoff) 735
Phosphorus, rickets, vitamin D effect, radioactive isotopes as indicator (Cohn and Greenberg) xvi
Potassium, radioactive isotopes as indicator (Joseph, Cohn, and Greenberg) 673
Protein (Rittenberg, Schoenheimer, and Keston) 603
Sterol (Treadwell and Eckstein) cv
Sulfanilamide, cystinuria (Kassell, Pearlstein, Brand, and Cahill) p. l
Tissue protein, pregnancy and lactation (Poo, Lew, and Addis) 69

Metabolism—*continued:*
Tyrosine, vitamin C relation (Sealock, Ziegler, and Driver) lxxxix

Metals: Liver oxidations, action (Bernheim and Bernheim) 79

Methemoglobin: Hemoglobin-, oxidation-reduction potentials, urea effect (Taylor) cii

Methionine: (Toennies and Kolb) 399
-Free diet, homocystine plus choline, growth effect (du Vigneaud, Chandler, Moyer, and Keppel) cviii
Oxidation (Toennies and Callan) civ

Methionine sulfoxide: *dl*- (Toennies and Kolb) 399

Methylcholanthrene: Growth effect (White and White) cx

Milk: Calcium, ionized (Nordbö) 745
Magnesium, ionized (Nordbö) 745

Mineral: Metabolism, radioactive isotopes in study (Joseph, Cohn, and Greenberg) 673
(Cohn and Greenberg) xvi

Morphine: Addicts, blood concentration (Williams) cxi

Mosaic: Tobacco, virus nucleic acid, diffusion constant and molecular weight (Loring) lxi
— — protein, amino acids (Ross and Stanley) lxxxiv

Mosaic—*continued:*
  Virus protein, latent, ultracentrifugal analysis (WYCKOFF) 729
Mucilage: Flaxseed, aldobionic acid structure (TIPSON, CHRISTMAN, and LEVENE) 609
Mucopolysaccharide: Synovial fluid, isolation (MEYER, SMYTH, and DAWSON) 319, lxx
Muscle: Cardiac, creatine, phosphorus, and potassium (MANGUN and MYERS) lxiii
  Dystrophy, nutritional, muscle phosphorus (GOETTSCH, LONSTEIN, and HUTCHINSON) 9
  Electrolytes, blood and, exchange, adrenal insufficiency (MUNTWYLER, MAUTZ, MANGUN, and MELLORS) lxxiv
  Phosphorus, muscle dystrophy, nutritional (GOETTSCH, LONSTEIN, and HUTCHINSON) 9
  Salts (LOWRY) lxii
  Voluntary, creatine, phosphorus, and potassium (MANGUN and MYERS) lxiii
  Water (LOWRY) lxii
  —, blood and, exchange, adrenal insufficiency (MUNTWYLER, MAUTZ, MANGUN, and MELLORS) lxxiv
  —, renal impairment influence (EICHELBERGER) 137
  Working, carbohydrate (FLOCK and BOLLMAN) xxx
  See also Exercise, Work

N

Neoplasm: Phospholipid metabolism, radioactive phosphorus as indicator (JONES, CHAIKOFF, and LAWRENCE) 631
Nephrectomy: Calciferol effect (TWEEDY, TEMPLETON, PATRAS, McJUNKIN, and McNAMARA) 407
Nephritis: Kidney oxidations (LYMAN and BARRON) lxii
Nicotinic acid: Determination, dysentery organism use in (DORFMAN, HORWITT, KOSER, and SAUNDERS) xx
Nucleic acid: Tobacco mosaic virus, diffusion constant and molecular weight (LORING) lxi
  Yeast, diffusion constant and molecular weight (LORING) lxi

O

Odocoileus virginianus borealis: *See* Deer
Oils: *See also* Fish liver oils, Olive oil, Tissue oil
Olive oil: Blood serum phosphorus, ingestion effect (REISER and HANES) lxxxii
  Urine phosphorus, ingestion effect (REISER and HANES) lxxxii
Organic material: Isotopes, radioactive, determination (CHARGAFF) 579, xiii
Organs: Protein metabolism, pregnancy and lactation (POO, LEW, and ADDIS) 69

Oxidase(s): Copper-protein, properties (McCarthy, Green, and King) 455
Xanthine, purification and properties (Ball) 51
Oxidation: Catalysts, tissue, copper and iron effect (Schultze) lxxxviii
Thermostable, tumor tissue (Feinstein and Stare) xxviii
Oxodelphinine: (Jacobs and Craig) 431
Oxonitine: Chemical constitution (Jacobs, Elderfield, and Craig) 439
Oxygen: Blood, influence (Cohn, Tannenbaum, Thalhimer, and Hastings) 109

P

Pancreas: Duct ligation, liver lipids, effect (Montgomery, Entenman, and Chaikoff) 387
Pancreatectomy: Protein conversion to glucose, effect (Gray, Ivy, and Cuthbert) 173
Parathyroid: Thyroid interrelation (Baumann and Sprinson) vi
Parathyroidectomy: Thyro-, calciferol effect (Tweedy, Templeton, Patras, McJunkin, and McNamara) 407
Peptides: Di-, racemization, acetylation with ketene (Cahill) xii
Multivalent (Greenstein) 241
Phenylalanine: Determination, reactions in (Block and Bolling) viii
Phlorhizin: Diabetes, pituitary, anterior, growth preparation, effect (Gaebler and Zimmerman) xxxii
Protein conversion to glucose, effect (Gray, Ivy, and Cuthbert) 173
Phosphatase: Bone, adenosinetriphosphate hydrolysis (Hitchings and Fuller) xlv
—, temperature effect (Bodansky) x
Venom, adenosinetriphosphate hydrolysis (Hitchings and Fuller) xlv
Phosphate: Liver, glycogen relation (Fenn) 297
Work capacity, effect (Morse) lxxiii
Phospholipid: Fatty livers (Flock, Hester, and Bollman) 153
Metabolism, liver, cholesterol influence, radioactive phosphorus as indicator (Perlman and Chaikoff) 735
—, neoplastic tissues, radioactive phosphorus as indicator (Jones, Chaikoff, and Lawrence) 631
—, radioactive phosphorus as indicator (Jones, Chaikoff, and Lawrence) 631
(Perlman and Chaikoff) 735
Phosphorus: Blood serum, olive oil ingestion, effect (Reiser and Hanes) lxxxii

Phosphorus—*continued:*
Dietary, hypervitaminoses D_2 and D_3, effect (MORGAN, HENDRICKS, and SHIMOTORI) lxxii
—, lead poisoning, influence (SOBEL, YUSKA, PETROVSKY, and KRAMER) xcvi
-Low diet, urinary calculi, relation (SCHNEIDER and STEENBOCK) lxxxvii
— —, vitamin D_2 effect (SCHNEIDER and STEENBOCK) 159
Metabolism, rickets, vitamin D effect, radioactive isotopes as indicator (COHN and GREENBERG) xvi
Muscle, cardiac and voluntary (MANGUN and MYERS) lxiii
—, muscle dystrophy, nutritional (GOETTSCH, LONSTEIN, and HUTCHINSON) 9
Radioactive, phospholipid metabolism indicator (JONES, CHAIKOFF, and LAWRENCE) 631
(PERLMAN and CHAIKOFF) 735
—, — —, neoplastic tissues, indicator (JONES, CHAIKOFF, and LAWRENCE) 631
Urine, olive oil ingestion, effect (REISER and HANES) lxxxii
Vitamin D determination, effect (O'BRIEN and MORGAREIDGE) lxxv

Photosynthesis: *Chlorella pyrenoidosa*, inhibition (GREEN, McCARTHY, and KING) 447

Pigment(s): Blood, abnormal, sulfanilamide relation (WENDEL and WENDEL) cix
Melanin, precursors, metabolism, vitamin C relation (SEALOCK, ZIEGLER, and DRIVER) lxxxix
Pyrrole-containing, hemoglobin synthesis, relation (KOHLER, ELVEHJEM, and HART) 501

Pituitary: Anterior, growth preparation, metabolism, phlorhizin diabetes, effect (GAEBLER and ZIMMERMAN) xxxii
—, hyperfunction (HARNED and COLE) xxxix
—, sperm whale, hormone (TOLKSDORF and JENSEN) cv
—. *See also* Antuitrin
Follicle-stimulating hormone, chemistry (FEVOLD) 83
Luteinizing hormone, chemistry (FEVOLD) 83

Plant: Green, amide metabolism (VICKERY and PUCHER) 703
—, — synthesis, mechanism (VICKERY and PUCHER) 703

Pneumococcus: Cinchona series, chemical constitution, relation (CRETCHER) xviii
Infection, 2-sulfanilamidopyridine effect (SCHMIDT) lxxxvi
Type XIV, polysaccharide, capsular, blood specific substance, relation (GOEBEL, BEESON, and HOAGLAND) xxxiv

Pneumonia: Blood, oxygen and carbon dioxide effect (Cohn, Tannenbaum, Thalhimer, and Hastings) 109
Sulfapyridine and related compounds, effect (Raiziss, Severac, Moetsch, and Clemence) lxxxi
Polycythemia: Blood cell, red, stroma, composition (Hoffman and Beach) xlv
Polysaccharide: Mucopoly-, synovial fluid, isolation (Meyer, Smyth, and Dawson) 319, lxx
Pneumococcus Type XIV, blood specific substances, relation (Goebel, Beeson, and Hoagland) xxxiv
Potassium: Distribution, intravascular injection, effect (Wilde) 309
Liver, glycogen relation (Fenn) 297
Metabolism, radioactive isotopes as indicator (Joseph, Cohn, and Greenberg) 673
Muscle, cardiac and voluntary (Mangun and Myers) lxiii
Pregnancy: Glucose kidney threshold (Gibson, Findlayson, and Paul) xxxiii
Organ and tissue protein, metabolism (Poo, Lew, and Addis) 69
Urine estrogenic substances, conjugated, hydrolysis (Heard and Edson) xli
— gonadotropic hormone (Gurin, Bachman, and Wilson) 525
Pregnancy—*continued:*
Urine gonadotropic hormone carbohydrate (Gurin, Bachman, and Wilson) xxxvii
— — —, preparation (Gurin, Bachman, and Wilson) 525
Pregnanediol glucuronidate: Sodium, adreno-genital syndrome, excretion (Venning, Weil, and Browne) cvii
Progestin: Creatine-creatinine metabolism, relation (Koven and Beard) lvi
Prolactin: Crop sac response, sex difference (Bates and Riddle) v
Properties (White and Bonsnes) cix
Protein(s): Bean, soy, storage effect (Jones and Gersdorff) xlix
Blood serum, mental disease (Kondritzer) lv
— —, regenerated, diet effect (Murrill and Newburgh) lxxiv
Copper-, oxidases, properties (McCarthy, Green, and King) 455
Deaminized cystine (Hess and Sullivan) 93
Dietary, growth, liver fat and, relation (Griffith) xxxvi
—, hemoglobin formation, relation (Orten and Orten) lxxvii
Egg white, ultracentrifugal and electrophoretic analysis (Young) cxiv
Glucose, conversion, pancreatectomy and phlorhizin effect (Gray, Ivy, and Cuthbert) 173

Protein(s)—*continued:*
  -High diets, fat synthesis, fasting (LONGENECKER) 645
  Kidney (FERRY, FERRY, and CONNOLLY) xxix
  Metabolism (RITTENBERG, SCHOENHEIMER, and KESTON) 603
  Mosaic virus, latent, ultracentrifugal analysis (WYCKOFF) 729
  Organs, metabolism, pregnancy and lactation (POO, LEW, and ADDIS) 69
  Sulfhydryl groups (GREENSTEIN) 233, xxxv
  Tissues, metabolism, pregnancy and lactation (POO, LEW, and ADDIS) 69
  Tobacco mosaic virus, amino acids (ROSS and STANLEY) lxxxiv
  Tuberculin, preservation (SEIBERT and DUFOUR) xc
  Wheat, storage effect (JONES and GERSDORFF) xlix
  Wool, properties (STEINHARDT and HARRIS) xcix
Prothrombin: Blood, infant (QUICK) lxxx
Pyrrole: -Containing pigments, hemoglobin synthesis, relation (KOHLER, ELVEHJEM, and HART) 501

Q

Quinine: Blood, determination (KYKER and ANDREWS) lviii

R

Renal: *See* Kidney
Renin: Purification (HELMER and PAGE) xlii
Respiration: *Chlorella pyrenoidosa*, inhibition (GREEN, MCCARTHY, and KING) 447
Rhubarb: Leaf, excised, carbon loss during culture (VICKERY and PUCHER) 685
Riboflavin: Vitamin A utilization, relation (KIMBLE and GORDON) lii
  See also Vitamin B_2
Rickets: Phosphorus metabolism, vitamin D effect, radioactive isotopes as indicator (COHN and GREENBERG) xvi
Roentgen ray: Diffraction membranes (SPIEGEL-ADOLF and HENNY) xcvii

S

Salts: Muscle (LOWRY) lxii
Sarcoma: Lympho-, mammary, phospholipid metabolism, radioactive phosphorus as indicator (JONES, CHAIKOFF, and LAWRENCE) 631
  Mammary, phospholipid metabolism, radioactive phosphorus as indicator (JONES, CHAIKOFF, and LAWRENCE) 631
  180, mammary, phospholipid metabolism, radioactive phosphorus as indicator (JONES, CHAIKOFF, and LAWRENCE) 631
Schizophrenia: Blood serum choline esterase, insulin effect (RANDALL) lxxxii
  — — lipids, insulin effect (RANDALL) lxxxii
Sex: Crop sac response to prolactin, effect (BATES and RIDDLE) v

Skin: Cholesterol, ultraviolet irradiation effect (Knudson, Sturges, and Bryan) 721

Sodium pregnanediol glucuronidate: Adreno-genital syndrome, excretion (Venning, Weil, and Browne) cvii

Sorbitol: *d*-, fate (Carr and Forman) 425

Sorbose: *l*-, fate (Carr and Forman) 425

Soy bean: *See* Bean

Spermidine: Tissue oxidation, effect (Evans) xxv

Spermine: Tissue oxidation, effect (Evans) xxv

Starch: Cereal, stomach emptying rate, effect (Froeschle and Pierce) xxxi

Statistics: Biochemical variables, normal men (Jellinek and Looney) 621

Sterol(s): Chemical activation (Eck and Thomas) 257, 267
- — — and configuration, relation (Eck and Thomas) 257
- Color reactions, chemical activation (Eck and Thomas) 267
- Metabolism (Treadwell and Eckstein) cv

Stomach: Analysis, dilution indicator technique, errors (Hollander and Bandes) xlvii
- Emptying rate, cereal starch effect (Froeschle and Pierce) xxxi

Streptococcus hæmolyticus: β-, hemolysin, properties (Smythe and Harris) xciv

Strontium: Biochemistry (Andersch and Fay) iii

Styracitol: Fate (Carr and Forman) 425

Succinic dehydrogenase: -Cytochrome system, aluminum, chromium, and rare earths, effect (Horecker, Stotz, and Hogness) 251

Sucrose: Clearance (Power, Goudsmit, and Keith) lxxix

Sugar: Alcohols (Carr and Forman) 425

Sulfanilamide: Blood cholesterol, herpes, effect (Member and Bruger) lxix
- —, effect (Webb and Kniazuk) 511
- — pigment, abnormal, relation (Wendel and Wendel) cix
- Determination, coupling component (Bratton and Marshall) 537
- Metabolism, cystinuria (Kassell, Pearlstein, Brand, and Cahill) p. l

Sulfanilamidopyridine: 2-, pneumococcus, effect (Schmidt) lxxxvi

Sulfanilyl-2-aminopyridine: Urine excretion products (Ratish, Bullowa, Ames, and Scudi) 279

Sulfapyridine: Pneumonia, effect (Raiziss, Severac, Moetsch, and Clemence) lxxxi
- -Related compounds, pneumonia, effect (Raiziss, Severac, Moetsch, and Clemence) lxxxi

Sulfate: Clearance (Power, Goudsmit, and Keith) lxxix

Sulfhydryl groups: Edestin solutions (Greenstein) 233
  Excelsin solutions (Greenstein) 233
  Globin solutions (Greenstein) 233
  Proteins (Greenstein) 233, xxxv

Sulfur: -Containing amino acids, enzyme oxidation (Medes) lxvii

Sulfur dioxide: Vitamin B complex stability, effect (Smith and Dimick) xciii

Surface tension: Enzyme activity, relation (Marron and Moreland) lxiv

Synovial fluid: Mucopolysaccharide isolation (Meyer, Smyth, and Dawson) 319, lxx

T

Taurocholic acid: Production (Virtue and Doster-Virtue) 665
  —, cysteine, homocysteine, and thioglycolic acid effect (Virtue and Doster-Virtue) 665

Temperature: Body, environmental temperature effect (Hill and Schultz) xliv

Testosterone: *m*-Dinitrobenzene reaction compounds, absorption spectra (Langstroth and Talbot) 759

Thiamine: Body fat synthesis, effect (McHenry and Gavin) 45

Thiamine—*continued:*
  Dietary requirement (Arnold) iv
  Fatty livers, effect (Engel and Phillips) xxv
  Urine (Melnick and Field) lxviii
  See also Vitamin B_1

Thioglycolic acid: Taurocholic acid production, effect (Virtue and Doster-Virtue) 665

Thyroglobulin: Chemical constitution (Brand, Kassell, and Heidelberger) xi

Thyroid: Amino acid oxidation, kidney and liver, feeding effect (Klein) 659
  Hormone, blood, basal metabolism, relation (McClendon and Rice) lxv
  Parathyroid interrelation (Baumann and Sprinson) vi

Thyroidectomy: Amino acid oxidation, kidney and liver, effect (Klein) 659

Thyroparathyroidectomy: Calciferol effect (Tweedy, Templeton, Patras, McJunkin, and McNamara) 407

Tissue(s): Creatine-creatinine distribution and interrelationship (Miller, Baker, and Allinson) lxx
  Creatine determination, enzymatic (Miller, Baker, and Allinson) lxx
  Creatinine determination, enzymatic (Miller, Baker, and Allinson) lxx
  Fatty acids, carbon tetrachloride administration, effect (Winter) 283

Tissue(s)—*continued:*
  Lipids, Virginia white-tailed deer, composition (TREADWELL and ECKSTEIN) 373
  Maltose determination, glucose presence (KOEHLER, MARSH, and HILL) liii
  Oil, determination (PUGSLEY) lxxx
  —, vitamin A determination (PUGSLEY) lxxx
  Oxidation catalysts, copper and iron deficiencies, effect (SCHULTZE) lxxxviii
  —, spermine and spermidine effect (EVANS) xxv
  Protein metabolism, pregnancy and lactation (POO, LEW, and ADDIS) 69
  Tumor, oxidations, thermostable (FEINSTEIN and STARE) xxviii
  —, ultraviolet irradiation effect (KNUDSON, STURGES, and BRYAN) 721

Tobacco: Mosaic virus nucleic acid, diffusion constant and molecular weight (LORING) lxi
  — — protein, amino acids (ROSS and STANLEY) lxxxiv

Tuberculin: Protein, preservation (SEIBERT and DUFOUR) xc

Tumor(s): Diet effect (BAUMANN, JACOBI, and RUSCH) vi
  Endocrine influence (BISCHOFF, LONG, and RUPP) viii
  Liver cell, vitamin A relation (GOERNER and GOERNER) 559
  Tissue, oxidations, thermostable (FEINSTEIN and STARE) xxviii

Tumor(s)—*continued:*
  Tissue, ultraviolet irradiation effect (KNUDSON, STURGES, and BRYAN) 721

Tyrosine: Metabolism, vitamin C relation (SEALOCK, ZIEGLER, and DRIVER) lxxxix

U

Ultraviolet irradiation: Blood cholesterol, effect (KNUDSON, STURGES, and BRYAN) 721
  Skin cholesterol, effect (KNUDSON, STURGES, and BRYAN) 721
  Tumor tissue, effect (KNUDSON, STURGES, and BRYAN) 721

Ultraviolet radiation: Egg albumin denaturation and molecular splitting, effect (BERNHART) 289

Urea: Blood, chicken, determination (HOWELL) 573
  Formation, liver, normal and diabetic, insulin action (STADIE, LUKENS, and ZAPP) xcviii
  Hemoglobin-methemoglobin oxidation-reduction potentials, effect (TAYLOR) cii

Uric acid: Excretion, dietary carbohydrate and fat effect (ADLERSBERG and ELLENBERG) 379
  —, inheritance (TRIMBLE and KEELER) cvi

Urine: Calculi, phosphorus-low diet, relation (SCHNEIDER and STEENBOCK) lxxxvii
  Estrogenic substances, conjugated, pregnancy, hydrolysis (HEARD and EDSON) xli

Urine—*continued:*
Gonadotropic hormone carbohydrate, pregnancy (GURIN, BACHMAN, and WILSON) xxxvii
— —, male, assay (HELLER, HELLER, and SEVRINGHAUS) xli
— —, pregnancy, preparation (GURIN, BACHMAN, and WILSON) 525
Maltose determination, glucose presence (KOEHLER, MARSH, and HILL) liii
Phosphorus, olive oil ingestion, effect (REISER and HANES) lxxxii
Pregnancy, gonadotropic hormone (GURIN, BACHMAN, and WILSON) 525
Sulfanilyl-2-aminopyridine, excretion products (RATISH, BULLOWA, AMES, and SCUDI) 279
Thiamine (MELNICK and FIELD) lxviii
Vitamin C (LONGENECKER, MUSULIN, and KING) lx
— —, determination (ROE and HALL) 329

V

van den Bergh reaction: (COOLIDGE) xvii
Venom: Phosphatase, adenosinetriphosphate hydrolysis (HITCHINGS and FULLER) xlv
Virus: Mosaic protein, latent, ultracentrifugal analysis (WYCKOFF) 729

Virus—*continued:*
Tobacco mosaic, nucleic acid, diffusion constant and molecular weight (LORING) lxi
— —, protein, amino acids (ROSS and STANLEY) lxxxiv
Vitamin(s): A, cyclized, fish liver oils (EMBREE) 187
— —, fish liver oils, nature (HICKMAN) xliii
—, goiter from low iodine intake, effect (HARRIS and REMINGTON) xl
—, liver cell tumors, relation (GOERNER and GOERNER) 559
—, tissue oil, determination (PUGSLEY) lxxx
—, utilization, riboflavin and ascorbic acid relation (KIMBLE and GORDON) lii
B, body fat, effect (McHENRY and GAVIN) lxvi
— complex, Factor W relation (FROST and ELVEHJEM) 23
— — stability, sulfur dioxide and autoclaving (SMITH and DIMICK) xciii
—, fat metabolism, relation (McHENRY and GAVIN) 45
—, liver fat, effect (McHENRY and GAVIN) lxvi
—. *See also* Filtrate factor
B_1, blood cholesterol, herpes, effect (MEMBER and BRUGER) lxix
—, body fat, effect (SCHLUTZ and OLDHAM) lxxxvi
—, iron retention, effect (SCHLUTZ and OLDHAM) lxxxvi
—. *See also* Thiamine

Vitamin(s)—*continued:*
  B_2. *See also* Riboflavin
  B_6, acrodynia, relation (DANN) xviii
  C, melanin pigment precursors, metabolism relation (SEALOCK, ZIEGLER, and DRIVER) lxxxix
  —, urine (LONGENECKER, MUSULIN, and KING) lx
  —, —, determination (ROE and HALL) 329
  D determination, phosphorus effect (O'BRIEN and MORGAREIDGE) lxxv
  —, dietary, lead poisoning, influence (SOBEL, YUSKA, PETROVSKY, and KRAMER) xcvi
  —, phosphorus metabolism, rickets, effect, radioactive isotopes as indicator (COHN and GREENBERG) xvi
  D_2, phosphorus-low diet, effect (SCHNEIDER and STEENBOCK) 159
  Deficiency, anemia, chick, relation (HOGAN and PARROTT) xlvi
  See also Grass juice factor, Hypervitaminosis, Hypovitaminosis

W

Water: Alcohol determination, aerometric (HARGER, BRIDWELL, and RANEY) xxxviii
Water—*continued:*
  Kidney (EICHELBERGER and BIBLER) xxiv
  Muscle (LOWRY) lxii
  — and blood, exchange, adrenal insufficiency (MUNTWYLER, MAUTZ, MANGUN, and MELLORS) lxxiv
  —, renal impairment influence (EICHELBERGER) 137
Whale: Sperm, pituitary, anterior, hormone (TOLKSDORF and JENSEN) cv
Wheat: Proteins, storage effect (JONES and GERSDORFF) xlix
Wool: Amino acids (BLOCK) 181
  Protein, properties (STEINHARDT and HARRIS) xcix
Work: Muscle carbohydrate (FLOCK and BOLLMAN) xxx
  Phosphates, effect (MORSE) lxxiii
  See also Exercise

X

Xanthine: Oxidase, purification and properties (BALL) 51
X-ray: *See* Roentgen ray

Y

Yeast: Nucleic acid, diffusion constant and molecular weight (LORING) lxi

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

THIRTY-THIRD ANNUAL MEETING

Toronto, Canada, April 26–29, 1939

(Received for publication, March 6, 1939)

The Biochemistry of Strontium.* By MARIE ANDERSCH AND MARION FAY. *From the Laboratory of Physiological Chemistry, Woman's Medical College of Pennsylvania, Philadelphia*

Adult dogs on the Cowgill diet† were fed (1) single doses and (2) repeated doses of strontium lactate. The total calcium and strontium and the filtrable calcium and strontium of the blood serum were determined according to the method of Sobel and his associates.‡ Analyses of the urine and feces were also made. A single dose of 25 mg. of strontium per kilo of body weight was excreted and stored so rapidly that often no strontium was detectable in the serum at the end of 24 hours. Repeated doses of the same size also disappeared from the serum in the same length of time. The level of calcium in the serum seems to bear no relationship to the amount of strontium; the former may remain at a normal level or may be reduced in the presence of strontium. The filtrable strontium is always low and often entirely absent, even when the total strontium is fairly high. Serum phosphate seems to be unaffected by the presence of strontium. Great irregularity in the excretion of calcium and strontium even on a constant intake has been observed. Bone analyses showed extensive storage of strontium. After parathyroidectomy this stored strontium is released into the blood, preventing the onset of tetany. Thus in one animal no symptoms of tetany were apparent 1½ months after all medication had ceased, even though calcium values in the serum were well below tetany levels. Severe tetany had followed the operation and careful pathological examination revealed no parathyroid regeneration.

Studies on Cystinuria; the Effect of Diuresis on the Excretion of Cystine by the Cystinuric. By JAMES C. ANDREWS AND KATHLEEN CRANDALL ANDREWS. *From the Department of*

---

* Aided by a grant from the Committee on Scientific Research, American Medical Association.

† Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).

‡ Sobel, A. E., Pearl, A., Gerchick, E., and Kramer, B., *J. Biol. Chem.*, **118**, 47 (1937).

Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, and the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill

The effect of the administration of large quantities of water on the excretion of cystine by a cystinuric subject has been studied under conditions of rigid dietary control. This study was made in conjunction with experiments on the administration of methionine to this subject and was undertaken to determine the importance of careful control of daily urine volume in such experiments.

In each of two such experiments, with a rigidly controlled diet, the 24 hour urine volume was brought to nearly 4000 ml. by copious water drinking. In one case, with a urine volume of 3700 ml., the output of cystine was increased by about 40 per cent over the basic level obtained with urine volumes averaging about 900 ml. In the other experiment, with a urine volume of nearly 4000 ml., the increase in cystine output amounted to over 50 per cent.

Administration of 5 gm. of methionine on each of two successive days, under the same conditions of dietary control as in the diuresis experiment but with a normal volume of urine, raised the cystine output about 30 per cent over the basic level.

The daily urine volume of the subject is obviously a matter of prime importance in the interpretation of such experiments.

Influence of the Composition of the Diet on the Thiamine Requirement of Dogs. By Aaron Arnold. *From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

Studies with rats and chicks from this laboratory have demonstrated that the thiamine requirements for normal growth are satisfied by the addition of approximately 80 microgram per cent of thiamine chloride to the basal ration. An extension of these studies to the nutrition of dogs has demonstrated that growth and protection from polyneuritis result when the ration is supplemented with approximately 75 microgram per cent of thiamine chloride. The addition of 50 microgram per cent of thiamine chloride to the ration does not prevent the onset of the anorexia associated with a thiamine deficiency. The ration used in the studies with the dogs had the following composition: sucrose 72, purified casein 18, autoclaved yeast 4, steamed bone meal 2, Salts I* 2, cottonseed oil 1, cod liver oil 1, and a sulfited liver extract supplement.

The daily requirement was markedly reduced by the isocaloric substitution of autoclaved lard for the sucrose in the above ration. When the ration contained approximately 57 per cent fat, supplied by the autoclaved lard, cod liver oil, and cottonseed oil, 27 microgram per cent of the thiamine chloride in the ration supported growth.

These results indicate that the thiamine requirement is best expressed on the basis of the non-fat constituents of the diet.

The Question of a Sex Difference in Crop Sac Response to Prolactin.

By Robert W. Bates and Oscar Riddle. *From the Carnegie Institution of Washington, Station for Experimental Evolution, Cold Spring Harbor*

Folley and White reported that subcutaneously injected prolactin produced heavier crop sacs in male than in female pigeons, but with simultaneous estrogenic hormone treatment crop sac weight responses were reduced and the sex difference nearly eliminated. Upon repeating their experiments, we found that equal amounts of prolactin regularly produced heavier crop sacs in male Carneau pigeons than in females, with or without estrogenic hormone treatment.

Seven groups of Carneau pigeons (ten to fifteen in each group) were injected subcutaneously with prolactin daily during 4 days. Three of these groups received no other treatment, three groups received dihydroestrone, and one group was fasted during the 4 days of treatment. At autopsy the crop sacs were weighed and the weights converted into equivalent prolactin units. The individual group quotients ((units from male glands)/(units from female glands)) were found to be 1.6, 6.0, 4.0, 2.4, 3.0, 2.4, and 2.4; average quotient for forty-eight males and thirty-two females 3.1. Birds injected intramuscularly (thirty-nine males and thirty-four females) had an average quotient of only 1.4. This quite inexplicable sex difference is seen to be greater after subcutaneous than after intramuscular injection. The sex difference observed with intramuscular injection, specified in our method of assay, is scarcely significant.

---

* Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).

Simultaneous treatment with dihydroestrone (a single injection of 10 mg.) reduced the crop sac response to prolactin to the level found in hypophysectomized pigeons, or, in terms of units, to about one-eighth that of normal pigeons.

The Effect of Diet on Tumors Produced in Various Ways.

BY C. A. BAUMANN, H. P. JACOBI, AND H. P. RUSCH. *From the Departments of Biochemistry and Physiology, University of Wisconsin, Madison*

Five diets were fed to mice receiving various treatments known to result in tumor formation. The diets were the Steenbock stock ration, a high fat diet, a high liver diet, a high cholesterol diet, and a diet containing a brain concentrate. The diets were also fed to tumor-bearing animals.

Tumors induced by ultraviolet light, or by painting with benzpyrene, showed the greatest sensitivity to diet, tumors induced by the injection of carcinogenic agents showed slight sensitivity, whereas preformed tumors, either primary or inoculated, were unaffected. The high fat diet markedly accelerated the production of epitheliomas by ultraviolet or benzpyrene painting, but fat did not accelerate the production of sarcomas. The brain concentrate showed a slight tendency to retard tumor production with most of the carcinogenic procedures tried.

Interrelation of the Thyroid and Parathyroid Glands.

BY EMIL J. BAUMANN AND DAVID B. SPRINSON. *From the Laboratory Division, Montefiore Hospital, New York*

Since the controversy over the possible anatomical relationship of the thyroid and parathyroid glands has been settled, these organs have been regarded as quite distinct anatomically and physiologically. Yet during the last decade the thyroid has been found to exert an important influence on calcium and phosphorus metabolism, though it is entirely different from that exercised by the parathyroids. In this report we shall present evidence that thyroid deficiency in rabbits brings about an increase in the amount of circulating parathyroid hormone, as shown by the Hamilton and Schwartz test, equivalent to that produced by injection of 10 to 30 Hansen units of parathyroid extract per kilo. Thyroidectomized male rabbits give a stronger response to the Hamilton test than do females. Injection of testosterone in male rabbits also brings about a greater response to this test. An interrelation of the thyroid and parathyroid glands, in which the testes may also take part, is thus indicated.

The Biological Relation of Creatine and Creatinine. By HOWARD H. BEARD, A. LINK KOVEN, AND PHILIP PIZZOLATO. *From the Department of Biochemistry, School of Medicine, Louisiana State University, New Orleans*

Evidence has already been presented to show that creatinine injection in young rats increases the content of muscle creatine and greatly stimulates the excretion of creatine and creatinine in the urine. The injection of creatine caused a retention instead of an increased excretion of creatinine. Similar results were obtained on four normal subjects. The chromogenic material (total creatinine) in all these urines was completely destroyed by the specific creatinine enzyme of Miller and Dubos; there is therefore no doubt that there was an increased excretion of creatine in these cases. For this reason it may be assumed that creatinine is hydrolyzed to creatine in the body.

Further evidence is presented showing the following results: injection of 2 to 10 cc. of saline, extra creatine excretion 32 to 56 mg., average 41 mg.; injection of 1 cc. of saline with 20 to 200 mg. of creatinine, extra creatine excretion 29 to 70 mg., average 43 mg.; injection of 2 to 10 cc. of saline with 20 to 200 mg. of creatinine, extra creatine excretion 67 to 101 mg., average 85 mg. The injection of saline and creatinine therefore gives the same average excretion of creatine as the sum of each injected alone. No decreases in muscle creatine occurred. Ingestion of 2000 cc. of water containing 10 and 20 gm. of NaCl, in addition to the usual water intake, during a 2 day period by two subjects resulted in an excretion of 4.4 and 9.6 gm. of creatine, respectively, in the urine.

Endocrine Factors Influencing Tumor Development. By Fritz Bischoff, M. Louisa Long, and J. Jerome Rupp. *From the Chemical Laboratory, Santa Barbara Cottage Hospital, Santa Barbara*

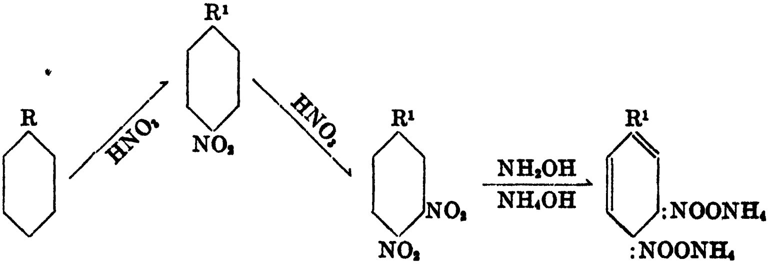
Our strain of virgin Marsh-Buffalo mice develops a high incidence (80 per cent) of mammary gland adenocarcinoma which is endocrine-linked, depending upon the presence of the ovaries. Groups of thirty to 50 of these mice received the following hormones subcutaneously; the dosage was begun before the attainment of sexual maturity: prolan per mouse, 60 rat units per month, 4 day period, 135 rat units per month, 4 day period, 300 rat units per month, 10 day period; theelin per mouse, 400 Doisy units per month, semiweekly, 800 Doisy units per month, semiweekly, 800 Doisy units alternate months, semiweekly; gonadin per mouse, 50 to 100 rat units per month, 10 day period. Equal numbers of litter mates served as controls.

Contrary to the current concept that theelin increases carcinogenesis in susceptible mice, neither the theelin nor the prolan, which obviously stimulates theelin-like production endogenously, increased the incidence of mammary tumor formation. Gonadin significantly delayed the onset of carcinogenesis, without producing functional castration and actually stimulated acinous development in the mammary gland. Prolan likewise produced acinous development (previously reported) but did not delay or enhance carcinogenesis or produce functional castration. Male mice given theelin did not develop breast tumors. It is concluded that the Marsh-Buffalo adenocarcinoma of the breast is linked to the ovary by some other secretion than theelin. Gonadin changes the ovarian secretion to produce substances which stimulate normal acinous development without producing carcinogenesis.

Chemical Reactions Involved in the Determination of Phenylalanine. By Richard J. Block and Diana Bolling. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York*

When phenylalanine is warmed with concentrated sulfuric acid containing 20 per cent by weight of potassium nitrate and the resulting nitration products are treated with hydroxylamine in alkaline solution, a blue-violet color is formed which is proportional to the amount of phenylalanine.

In an effort to elucidate the mechanism of this reaction, 3,4-dinitrotoluene, 2,3-dinitrotoluene, 2,5-dinitrotoluene, 2,6-dinitrophenol, 3-nitro-4-nitrosotoluene, 2-nitroso-3-nitrotoluene, 2,4-dinitrobenzoic, 3,5-dinitrobenzoic, *o*-, *m*-, and *p*-nitrobenzoic, phenylacetic, hydrocinnamic, cinnamic, mandelic, and phenylpyruvic acids, and nineteen other compounds were investigated. The results indicate that the chromogen appears to be primarily a derivative of diaci-3,4-dinitrodihydrobenzene with the possibility of a small amount of diaci-2,3-dinitrodihydrobenzene. The reaction may take the following course.



$R = CH_2CHNH_2COOH$; R^1 may be R, $CH_2CH(NH \cdot NO_2)COOH$.

Under the conditions observed, phenylalanine gives approximately 25 per cent of the color produced by an equimolar amount of 3,4-dinitrotoluene.

The Determination of Adrenalin in Blood. By W. R. Bloor.

From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York

The chemical methods for adrenalin which approach closely the requirements for its determination in blood are those of Whitehorn (1935) and its modification by Shaw* (1938). Shaw substitutes a double adsorption by $Al(OH)_3$, first at pH 4 then at pH 8.5 for Whitehorn's single adsorption on silica which does not extract the adrenalin completely. In the present method,

---

* Whitehorn, J. C., *J. Biol. Chem.*, **108**, 633 (1935). Shaw, F. H., *Biochem. J.*, **32**, 19 (1938).

Shaw's differential adsorption is found excellent and retained. The arsenomolybdate reagent was found much more sensitive (and less stable) without the addition of bromine water and is so used, but its use requires occasional redrawing of the calibration curve. In the determination, the second $Al(OH)_3$ precipitate carrying the adrenalin is dissolved in the arsenomolybdate reagent and digested for about 5 minutes in boiling water, after which 3 cc. of the sulfite-sulfuric mixture is added and the heating continued for 10 minutes. The predigestion with the reagent results in the production of more color and renders the whole reaction more certain. The increase of heating time to 10 minutes followed a study of the development of the color, which reaches its maximum after not less than 8 minutes heating. A photoelectric colorimeter is used for the measurement of the color. In adrenalin solutions, the color from 0.01 microgram can be measured to about 1 per cent (0.0001 microgram). Determinations on blood can be repeated within 5 per cent. The level found for human blood is about 0.05 microgram per cc.

Effect of Temperature on Activity of Bone Phosphatase. By Oscar Bodansky. *From the Children's Medical Service, Bellevue Hospital, and the Department of Pediatrics, New York University College of Medicine, New York*

The effect of temperature on the activity of bone phosphatase has been studied at optimal pH (9.0 to 9.2) and under optimal conditions of magnesium concentration (0.01 M) and of α-amino acid concentration (0.00625 M glycine). Inactivation of the enzyme during a 25 minute incubation period under these conditions occurred at temperatures above 42–43°. The velocities of the hydrolysis of 0.0127 M sodium β-glycerophosphate were determined at nine temperatures ranging from 12–42.4° in the case of cat bone phosphatase, and at five temperatures ranging from 20–40° in the case of human bone phosphatase. The observed velocities conformed with the linear relationship demanded by the Arrhenius equation. The values for *E*, the energy of activation, were 9870 ± 160 calories for the observations on cat bone phosphatase and 10,140 ± 270 calories for those on human bone phosphatase and did not, therefore, differ significantly from each other.

For the abstract by Henry Borsook and Jacob Dubnoff see p. cxiv.

On the Structure of Thyroglobulin.

By Erwin Brand, Beatrice Kassell, and Michael Heidelberger. *From the Departments of Biological Chemistry and of Medicine, College of Physicians and Surgeons, Columbia University, New York*

The molecular weight of thyroglobulin was recently determined by Heidelberger and Pedersen* in part on a dialyzed sample (iodine content 0.58 per cent), isolated from Swedish hog thyroids according to Heidelberger and Palmer.† The analysis of a denatured part of this same sample by methods developed in this Department gave the following results (in per cent of protein): total protein sulfur 1.45, sulfate sulfur 0.50, cystine 4.30, cysteine 0, methionine 1.31, tryptophane 1.88, tyrosine 3.00, diiodotyrosine 0.67, thyroxine 0.28, and glucosamine‡ 2.2 per cent.

With a value of 665,000 for the molecular weight of thyroglobulin (Heidelberger and Pedersen's determinations yielded an average of 675,000) and in the light of Bergmann's theory, the above results indicate that the thyroglobulin molecule contains 5760 amino acid residues, of which 120 are cystine (240 if considered as cysteine residues), 60 methionine, 60 tryptophane, 110 tyrosine, 10 diiodotyrosine, and 2 thyroxine. There are also present 80 glucosamine residues (in addition to other carbohydrate). It appears possible that, aside from the special thyroxine grouping, the molecule of this protein hormone is composed of ten units of 576 amino acids each, the individual units being otherwise similar in amino acid composition.

The Use of Bromide As a Measure of Extracellular Fluids.

By Bernard B. Brodie, S. Leshin, and Elliott Brand. *From the Pharmacological Laboratory, College of Medicine, New York University, New York*

The volume of the extracellular fluids can be measured by estimating the serum concentration of administered substances which are diffusible, unaltered by the body, and which, like chloride, do not enter body cells. Thiocyanate is commonly used for this purpose, but the use of this substance suffers from the following disadvantages: (*a*) the inaccuracy of the urine analyses, (*b*) anomalous distribution between serum and transudates.

---

* Heidelberger, M., and Pedersen, K. O., *J. Gen. Physiol.*, **19**, 95 (1935).

† Heidelberger, M., and Palmer, W. W., *J. Biol. Chem.*, **101**, 433 (1933).

‡ For this determination, carried out on another sample of thyroglobulin, we are indebted to Dr. H. E. Stokinger.

Previous work in this laboratory has shown the similarity of distribution of chloride and bromide. Bromide is distributed quickly throughout the body and the excretion on the administration of small doses is slow. This insures uniform diffusion throughout all extracellular fluids. The determination can be made by a previously reported method, relatively simple, and accurate (within 1 per cent) on 1 ml. of serum or urine containing 0.1 mg. of bromide. Sufficient bromide remains in the body for 48 hours or more, so that if repeated determinations are necessary no more of the substance need be administered.

30 to 50 mg. of bromide per kilo are administered by stomach as the sodium or potassium salt. This produces no ill effects. In 3 hours or more, blood and urine samples are taken. In the 3 hour period the excretion of bromide is less than 1 per cent, and can therefore be neglected.

Bromide distributes between serum and transudates according to the predictions of the Gibbs-Donnan equilibrium. Therefore the following best represents the volume of extracellular fluid: ((amount of bromide administered minus amount excreted) $\div$ (concentration in serum water)) $\times$ 0.95.

Racemization of Amino Acids and Dipeptides on Acetylation with Ketene. By William M. Cahill. *From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*

In the course of an investigation of the synthesis of abrine Cahill and Jackson* had occasion to employ ketene as an acetylating agent. It was observed that under certain conditions a racemic acetylated derivative was obtained and a study of this racemization was subsequently made.† The present investigation was undertaken to extend the study to other representative amino acids and to dipeptides. *l*(−)-Leucine and *l*(+)-glutamic acid can be conveniently acetylated with ketene to give either the optically active or racemic N-acetyl derivative, depending upon the conditions employed. When *l*(+)-isoleucine is acetylated under racemizing conditions, the derivative secured is only partially racemized, owing apparently to the fact that the stereochemical uniformity of only one of the 2 asymmetric carbon atoms is destroyed by this procedure.

---

* Cahill, W. M., and Jackson, R. W., *J. Biol. Chem.*, **126**, 29 (1938).

† Jackson, R. W., and Cahill, W. M., *J. Biol. Chem.*, **126**, 37 (1938).

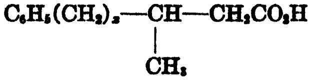
Both glycyl-*l*(−)-leucine and *l*(−)-leucylglycine can be readily acetylated with ketene to give the corresponding active acetyl derivatives. When acetylated under racemizing conditions, however, glycyl-*l*(−)-leucine yields an inactive N-acetyl product, whereas under the same conditions *l*(−)-leucylglycine yields the optically active acetyl derivative.

Metabolism of Fatty Acids.

By Herbert E. Carter, Elizabeth Osman, Harold Levine, and Stanford Gamm. *From the Laboratory of Biochemistry, University of Illinois, Urbana*

A study has been made of the metabolism of a series of ω-phenyl-β-alkyl fatty acids of the following types.

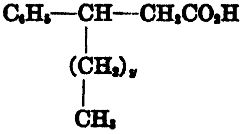
I.



$C_6H_5(CH_2)_x\text{—}\underset{\substack{|\\ CH_3}}{CH}\text{—}CH_2CO_2H$

$x = 0, 1, 2$

II.



$C_6H_5\text{—}\underset{\substack{|\\ (CH_2)_y\\ |\\ CH_3}}{CH}\text{—}CH_2CO_2H$

$y = 1, 2, 3, 4, 5$

The sodium salts of these acids were administered orally to dogs over a period of 3 to 5 days and the products were isolated from the urine by the usual methods. None of the acids fed yielded an appreciable amount of benzoic acid or phenylacetic acid. The compounds in (II) were excreted in the urine partly as the free acid and partly as the glycine conjugation product. The substances in (I) yielded varying amounts of unsaturated acid. Both the original acid and the unsaturated acid appeared in the urine in the free state and also conjugated with glycine.

The mechanism of oxidation of normal fatty acids and the metabolism of β-substituted fatty acids will be discussed in the light of these results.

The Determination of Radioactive Isotopes in Organic Material.

By Erwin Chargaff. *From the Department of Biological*

Chemistry, College of Physicians and Surgeons, Columbia University, New York

In the course of experiments on the enzymatic and metabolic formation of radioactive phosphatides and on the synthesis of radioactive organic substances containing the unstable isotope P^{32}, it was found desirable to determine the radioactivity of the compounds themselves rather than of their ignition residues. This can be effected in one of two ways: (1) The substances, dissolved in water or a suitable organic solvent, are contained in the hollow jacket of a double walled, thin glass cylinder, holding about 10 cc. of liquid, which is slipped over the counter tube of a Geiger-Müller counter. (2) The substances, in the dry state, are spread in a thin layer (thickness, approximately 0.5 mm.) on a small aluminum tray which can be arranged underneath the counter tube in one of three constant distances, thereby permitting the measurement of more or less active preparations, as the case may be. The second arrangement is preferable in the case of substances with low radioactivity. As standard a 50 per cent solution of potassium fluoride in water was chosen. The radioactivity of the preparations is expressed in KF units, which are defined as the amount of potassium fluoride in mg. which under comparable conditions shows the same degree of radioactivity as 1 mg. of the substance measured.

The Relative Speed of Formation of Lecithin and Cephalin in the Body. By Erwin Chargaff. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The biological function of the phosphatides in the body is still controversial. It has not yet been established whether the synthesis of the two principal groups of phosphatides, *viz.* the lecithins and the cephalins, in the animal organism follows the same course. When secondary sodium phosphate containing a small amount of the unstable isotope P^{32} is fed to adult rats and the radioactivities of the total lecithin and cephalin fractions are measured, it is found that the amounts of newly formed lecithin and cephalin are practically the same. 19 hours after the ingestion of $Na_2H^*PO_4$ the lecithin had 15.6, the cephalin 17.6 KF units (see the preceding abstract) per mg. of lipid P;

43 hours after feeding, the corresponding values were 23.7 units for the lecithin and 25.4 units for the cephalin. The study of the relative speed of formation of lecithin and cephalin in various organs, however, gives a different picture. 24 hours after the ingestion of $Na_2H^*PO_4$ containing a higher proportion of the unstable isotope than in the experiment quoted above the KF units per mg. of lipid P were found as follows: intestinal tract, lecithin 61.6, cephalin 35.4; liver, lecithin 81.3, cephalin 68.0; brain, lecithin 19.7, cephalin 23.2; carcass, lecithin 21.7, cephalin 22.1. The differences observed in the phosphatides from the intestinal tract and probably also in the liver phosphatides are significant. They may tend to show that the synthesis in the animal organism of lecithin and cephalin, at least in certain organs, does not follow the same course.

The Ionization of Barbituric Acids and Anesthetic Bases in Relation to Their Anesthetic Action. By G. H. A. Clowes, A. K. Keltch, and M. E. Krahl. *From the Lilly Research Laboratories, Indianapolis*

The concentrations of each of ten barbituric acids and each of fifteen local anesthetic bases required to produce anesthesia of *Arbacia* eggs and *Arbacia* larvæ were determined at three pH levels in carbonate-free sea water buffered with glycylglycine. From these data and the apparent dissociation constants determined for this purpose, the respective extracellular and intracellular concentrations of undissociated molecules corresponding to the total anesthetic concentrations were calculated. For each anesthetic, the extracellular concentration of undissociated molecules required to produce a given physiological effect was found to be the same at each of the three extracellular pH levels used. From these results it is tentatively concluded that all of the anesthetics here described penetrate *Arbacia* eggs and *Arbacia* larvæ only as undissociated molecules.

The concentrations of 5-isoamyl-5-ethylbarbituric acid and γ-(2-methylpiperidino)-propyl benzoate required to produce anesthesia in *Arbacia* eggs were also determined in the presence and absence of a partial pressure of carbon dioxide sufficient to effect an appreciable downward shift in the intracellular pH.

The extracellular concentration of the undissociated barbituric acid molecules required to produce a given effect was found to be the same, with and without this amount of carbon dioxide, indicating that, within the cells, the undissociated molecule is the active form of the barbituric acid.

The extracellular concentration of the undissociated local anesthetic molecules required to produce a given effect was diminished in the presence of the additional carbon dioxide, indicating that, within the cells, the cation is the active form of the local anesthetic base.

Studies in Mineral Metabolism with the Aid of Artificial Radioactive Isotopes. III. The Influence of Vitamin D on the Phosphorus Metabolism of Rachitic Rats. By Waldo E. Cohn and David M. Greenberg. *From the Division of Biochemistry, University of California Medical School, Berkeley*

With the aid of radiophosphorus (P^{32}), a study has been carried out on the influence of vitamin D on the phosphorus metabolism of rachitic rats. The following important effects were found to have been exerted by the administration of vitamin D to rachitic rats. The net absorption of phosphate, administered by stomach tube, from the gut was increased only about 10 to 15 per cent. The most striking result of the vitamin D administration was found in the phosphorus fractions of the bone. The alcohol-ether-soluble phosphorus (lipoid P) of fresh bone was not altered. The labeled phosphorus content in the inorganic form was raised about 40 per cent in both fat-free bone and bone ash. The $P^{32}:P^{31}$ ratio in the organic phosphorus fraction (protein and ester P) of the bone was increased and was always higher than in muscle. It became nearly equal to the ratio of $P^{32}:P^{31}$ in urine, which indicates that the organic phosphorus of the bone is rapidly formed from the blood phosphorus.

The influence of vitamin D on the deposition of inorganic phosphorus in the bone of rachitic rats must be ascribed to a more direct effect than one merely resulting from an increasing absorption. A specific effect of vitamin D on organic bone phosphorus and a specific rôle of this fraction in bone formation are indicated.

---

* We gratefully acknowledge our indebtedness to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for supplying us with the radiophosphorus.

The van den Bergh Reaction. By Thomas B. Coolidge. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

The "directness" of the van den Bergh reaction is not a function of the plasma cholic acid level as determined by analysis, or of any substance in the plasma in labile equilibrium with the bilirubin, as determined by the fact that mixtures of plasmas with different characteristics give rates of reaction which are the sums of the rates of reaction of the unmixed plasmas. Both directly and indirectly reacting bilirubin are attached to the albumin and the relative amounts are little altered by cataphoresis.

The function of the alcohol in the quantitative reaction is a purely catalytic one, as, after removal of the alcohol, the type of reaction is that which was originally present.

The bilirubin reacting immediately in aqueous solution is extractable by organic solvents.

Formation of Glycogen from Amino and Alkyl Derivatives of the Fatty Acids. By Ralph C. Corley and Mary Lyons. *From the Laboratory of Biochemistry, Department of Chemistry, Purdue University, Lafayette*

There is in progress an extensive study of the influence of substituent groups on the catabolism of various derivatives of the fatty acids. The present report concerns observations on the formation of liver glycogen in the white rat following the oral administration of aminated and alkylated fatty acids. Results with the α-amino derivatives of the fatty acids with straight chains and with 4, 5, 6, and 8 carbon atoms are in accord with the usual view that oxidative deamination with the loss of 1 carbon atom constitutes one stage in the degradation of α-amino acids. No simple explanation appears for the observation that β-aminobutyric acid is apparently a sugar former under the experimental conditions employed.

Structure and Antipneumococcic Activity in the Cinchona Series.

BY LEONARD H. CRETCHER. *From the Department of Research in Pure Chemistry, Mellon Institute, Pittsburgh*

Highly effective drugs of the cinchona series have been known, specifically active against the pneumococcus in high dilutions but impractical clinically because of toxicity. One especially unfortunate property has been that of producing blindness. But few generalizations can be made as to the relation of structure, bactericidal power, and toxicity. The rest of the molecule remaining unaltered, reduction of the vinyl group enhances activity, though shift of the double bond from vinyl to ethylidine produces the most powerful compounds found to date.

Specificity toward the pneumococcus has been thought to reside in the ethyl ethers. Further research, however, has demonstrated marked activity in ethers with longer and more complex side chains.

Active ethers of the simple cupreine or apoquinine structure are lessened in value if the bridge $\overset{|}{\underset{|}{\mathrm{CHOH}}}$ is changed to $\overset{|}{\underset{|}{\mathrm{CO}}}$ or to $\overset{|}{\underset{|}{\mathrm{CHCl}}}$ or $\overset{|}{\underset{|}{\mathrm{CH_2}}}$. The ethers of hydrocupreicine and hydrocupreicinol are also of little value.

Such fragmentary work as has been done would indicate that interference with the simple C and H nature of the side chain in the quinuclidine nucleus, whether by addition, oxidation, or otherwise, is not effective.

Variations in bactericidal power have been noted among stereoisomers. All effective compounds tested were considered too toxic until derivatives were made in which hydroxyl was introduced into the ether groupings. This has lowered toxicity and eliminated eye injury without destroying the desired effect.

Thus far the phenolic quinoline and quinuclidine rings bound together by a secondary alcohol group seem to be a necessary basis for specific pneumococcicidial action, compounds of quinoline not having proved satisfactory, as in malaria.

The Incidence of Acrodynia among Rats Receiving Diets Deficient in Vitamin B_6.

BY W. J. DANN. *From the Depart-*

ment of Physiology and Pharmacology, Duke University Medical School, Durham, North Carolina

Goldberger and Lillie reported in 1926 the occurrence of a characteristic symmetrical dermatitis (since named acrodynia) among rats given diets deficient in vitamin B_2. Other workers were unable to reproduce this symptom at all regularly, but when vitamin B_2 was shown by György and his coworkers to comprise two factors, flavin and vitamin B_6, it appeared that acrodynia was a symptom of deficiency of vitamin B_6 alone. György reported in 1935 that rats kept on a vitamin B_6-deficient diet developed acrodynia "almost without exception." This has not been the experience of the author, working in this laboratory. In the earliest experiments, begun in 1935, a majority of rats maintained on vitamin B_6-deficient diets developed acrodynia, but a considerable proportion failed to do so. In later experiments an increasing proportion of the rats has failed to develop this characteristic symptom.

Certain changes in the fat content of the diet or in the purity of the casein used were reported by other workers to render the appearance of acrodynia almost certain in a relatively short time, but had no effect in this laboratory. Similarly, control of environmental temperature and humidity failed to increase the incidence of acrodynia. In the author's experience the production of acrodynia among rats maintained on diets deficient in vitamin B_6 has not been susceptible to control. It is suggested that a refection phenomenon may be the disturbing agency.

A Simple Procedure of the Study of Fat Absorption. By Harry J. Deuel, Jr., Lois F. Hallman, and S. Quon. *From the Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles*

The fat is fed to the unanesthetized animal by stomach tube attached to the tuberculin syringe. At the appropriate time, the intact gastrointestinal tract is removed, from esophagus to rectum. The contents are separated from the gastrointestinal tract by forcing successively 50 cc. of water at 70°, 20 cc. of petroleum ether, and 20 cc. of water through the gut from a syringe connected to the esophagus. During this process the stomach and cecum are continuously massaged to remove any solid material.

After acidification of the gut contents, the fat is removed by extraction for 4 hours in a modified Kutscher-Steudel apparatus, with petroleum ether as a solvent. The quantity of fat extracted is determined from the increase in weight of the flasks after removal of the petroleum ether by evaporation and by drying under reduced pressure. When 1.5 cc. of cottonseed oil are administered to a rat and the animal is immediately killed, an average of 95 per cent is recovered. The control level for fasted rats is only 5 mg.

Metabolic Effects of Adrenalin and Benzedrine. By D. B. Dill, R. E. Johnson, and C. Daly. *From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston*

The cycle of changes in pulse rate, blood pressure, blood sugar, and blood lactate after the injection of 1 mg. of adrenalin chloride (Parke, Davis) was completed in 2½ hours or less. The R.Q. rose abruptly when lactate increased but did not fall correspondingly as lactate was removed; over the entire postinjection period the R.Q. was higher by 0.02 than before injection. Acetone bodies in blood and urine were not more concentrated than in experiments of like duration in which no drug was administered. The average calorigenic effect of adrenalin was +13 per cent; return to the metabolic level of the control experiments required about 2½ hours. Previous experiments have indicated that adrenalin favors carbohydrate utilization in exercise; these indicate that it does not interfere with carbohydrate utilization in normal man in the postabsorptive state.

The injection of 20 mg. of benzedrine sulfate (Smith, Kline and French) produced no change in blood sugar, blood lactate, or R.Q. and no consistent change in pulse rate. There was a gradual rise in blood pressure and in oxygen consumption throughout the 2½ hours of observation; during this time the average calorigenic effect was +7 per cent.

Benzedrine, although related structurally to adrenalin and classed with it as sympathomimetic, differs qualitatively and quantitatively in its action and disappears more slowly from the body.

The Use of the Dysentery Organism for the Quantitative Determination of Nicotinic Acid. By Albert Dorfman, M. K.

HORWITT, S. A. KOSER, AND FELIX SAUNDERS. *From the Departments of Biochemistry and of Bacteriology of the University of Chicago, Chicago, and the Elgin State Hospital, Elgin*

In a previous report we have shown that the dysentery bacillus is able to grow in a synthetic medium if 0.005 microgram per cc. of nicotinic acid is present. There is a close correspondence between the activity of nicotinic acid derivatives when tested as growth-promoting substances for the dysentery organism and as black tongue-preventing substances for dogs. Since no adequate chemical or biological method for the quantitative estimation of nicotinic acid and related compounds exists, we deemed it advisable to develop such a method, using the dysentery bacillus as a test organism.

The method consists essentially of determining the minimum amount of any unknown solution which when added to a synthetic medium will produce growth comparable to that obtained with a standard quantity of nicotinic acid. It is understood that we are measuring not only nicotinic acid, but also the other biologically occurring compounds which we have found active for this organism. These include nicotinamide, nicotinuric acid, and cozymase. Coenzyme II has not been tested.

The method has been applied to normal human blood, normal human urine, milk, and saliva. A remarkable constancy was found in human blood and in the daily urinary output. Almost all activity in blood has been found to be in the erythrocytes.

The Effect of Light Scattering upon the Absorption Spectra of Non-Hemolyzed Erythrocytes. By DAVID L. DRABKIN AND RICHARD B. SINGER. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The purpose of this study is the extension of spectrophotometry to the important field of very turbid systems, such as whole blood and tissues.

By means of a spectrophotometer ϵ values at different wavelengths were obtained for suspensions of non-hemolyzed dog erythrocytes, the number of red blood cells and the depth of the cuvette being varied. Suspensions of cream particles, with and without added HbO_2, were subjected to similar measurements.

The total light extinction by suspensions of red blood cells in the Drabkin and Austin cuvette* 0.07 mm. in depth could be accounted for by the equation, ϵ (total) = $f(N) \times \epsilon$ (HbO_2) + ϵ (scattering), where N is the number of cells per c.mm. and ϵ (scattering) represents the light lost owing to scattering by the cell particles in the suspension and possibly other as yet undefined factors. Under our conditions, ϵ (scattering) for both erythrocytes and cream particles was found to be a non-linear function of N, and independent of wave-length (between 630 and 500 mμ). At values of $N < 0.35 \times 10^6$, $f(N) < 0.1$, the absorption spectrum of HbO_2 was completely submerged by the non-specific "absorption" due to scattering. At N between 0.35×10^6 and 3.5×10^6, $f(N)$ varied between 0.1 and 1.0. At N = or $> 3.5 \times 10^6$, $f(N)$ became constant at a value of 1.0. Under the latter circumstances, the spectrum of HbO_2 within the erythrocytes was readily deduced.

The Function of Carbonate in the Synthesis of Glycine from Chloroacetic Acid, Ammonium Hydroxide, and Ammonium Carbonate.

By Max S. Dunn, A. W. Butler, and Edward H. Frieden. *From the Chemical Laboratory, University of California at Los Angeles, Los Angeles*

The rates of formation and yields of chloride and primary amine at three temperatures were measured. At equal concentrations of total ammonia, the yield of chloride is the same but that of primary amine is markedly increased with mixtures of ammonia and carbonate compared with ammonia alone. The rates of both reactions decrease as the equivalent ratio of carbonate to chloroacetic acid increases to 5:1 in mixtures containing total ammonia and chloroacetic acid in ratios less than 30:1.

The ratio of approximately 30:10:1 for total ammonia, total carbonate, and chloroacetic acid is optimum for the maximum production of primary amine. Glycine has been isolated repeatedly from such mixtures in yields which are 25 per cent higher than the 60 per cent of the theoretical yield obtainable with a 60:1 ratio of ammonia to chloroacetic acid, or an 8:1 ratio of ammonium carbonate to chloroacetic acid, as advocated by Robertson and Cheronis, respectively. The authors' method is more convenient and more economical than other procedures.

---

* Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 105 (1935-36).

The hypothesis is proposed that relatively high concentrations of both ammonia and carbonate promote a nearly complete amination of chloroacetic acid and largely eliminate side reaction products by forming carbaminoglycine from which glycine is subsequently regenerated. Consideration of the partial pressures of ammonia and carbon dioxide in the experimental systems supports the belief that the observed reactions are largely controlled by these factors.

Similar studies with homologous α-halogen acids are planned.

Biotic Acid; a New Growth Substance in Liver. By Robert E. Eakin and Roger J. Williams. *From the Chemical Laboratories of Oregon State College, Corvallis*

For the growth of yeast at a maximum rate from a small seeding, several substances which are as yet unknown must be present in the medium. This statement is true even if pantothenic acid (Williams *et al.*) and biotin (Kögl) are included among the "known" substances.

A new growth substance or nutrilite which supplements pantothenic acid, especially when tested on a pure culture from Fleischmann's yeast (pound goods), has been found in liver. It has been characterized as follows: It is an acid of about the same strength as pantothenic acid, and is readily oxidized anodically and by hydrogen peroxide. The molecular weight by diffusion is approximately 150. The methyl ester of the acid is practically as effective physiologically as the acid itself. The physiological activity is wholly destroyed by refluxing with alcoholic hydrochloric acid; in this respect, "biotic acid" behaves differently from biotin, which is unimpaired by this treatment. "Biotic acid" is highly water-soluble and is not adsorbed by charcoal, and hence according to the terminology of Miller would be bios II with some letter appended. The fractions of bios II are all so poorly characterized, however, that it is impossible to identify "biotic acid" with any previously recognized fraction.

The Survival of Cathepsin. By Howard Eder, H. C. Bradley, and Samuel Belfer. *From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison*

A study of the survival of cathepsin in hog liver digests indicates that at pH 2 the enzyme is destroyed immediately. At pH 2.5 it is completely inactivated in 5 days. At pH 3.0 much of the activity remains for 5 days. Between pH 3.5 and 5.0 the enzyme is quite stable for over 30 days. In such digests cathepsin shows a progressive increase of activity as autolysis proceeds for 10 days; by the end of 20 days there is evidence of gradual destruction. With increase of pH from 6 to 8 there is a gradual loss of cathepsin, in proportion to the alkalinity. Even at pH 8, however, activity persists for 20 days. At pH 9.0 it is destroyed quite rapidly. The rapid decrease of autolysis on the acid side of the optimum range appears referable to the diminishing enzyme mass. On the alkaline side, the decrease in autolysis appears referable to the mass of substrate as the limiting factor, although the activity of cathepsin itself in that pH region has not yet been definitely excluded.

Water and Electrolyte Content of Dog Kidneys. BY LILLIAN EICHELBERGER AND WALTER G. BIBLER. *From the Lasker Foundation for Medical Research, Department of Medicine of the University of Chicago, Chicago*

Total water, fat, and the concentrations of the principal electrolytes were determined in kidneys from dogs in the following groups: (1) twenty normal controls, whole kidneys; (2) five normal controls, in each of which one whole kidney was analyzed and the other kidney roughly dissected into medulla and cortex, which were analyzed separately; (3) ten hydronephrotic dogs, each having a single hydronephrotic kidney; (4) ten normal dogs, in each of which one kidney had been removed and the other kidney left for a period of 1 to 9 months.

Normal kidneys gave the following mean average results, which are expressed as units per kilo of fat-free kidney: total water, 800 gm., σ ±8.8; calcium, 2.5 mM, σ ±1.24; potassium, 58.6 mM, σ ±3.94; sodium, 84.0 mM, σ ±5.0; chloride, 69.1 mM, σ ±7.4. The right and left kidneys from the same animal gave the same analytical results. The values for water, fat, sodium, and chloride were always greater for the medulla than for the cortex.

The hydronephrotic kidneys differed from the normal in that there was an increase in total water content and a decrease in chloride concentration.

The single kidneys in normal dogs over varying periods up to 9 months did not differ in water and electrolyte concentration from the kidneys removed first. After the removal of one kidney from a normal dog, there was no hypertrophy of the remaining kidney within the period of observation (1 to 9 months).

Chemical and Histologic Studies of Fatty Livers Produced in Thiamine Therapy of Experimental Vitamin B_1 Deficiency.

By R. W. Engel and Paul H. Phillips. *From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

Thiamine administration in experimental vitamin B_1-deficient chicks or rats produces "fatty" livers. Chemical analyses of these livers show an increased deposition of glycogen and a slight but definite increase in alcohol-extractable substances. The total nitrogen in the residue after alcohol extraction remains unchanged. These chemical changes occur as soon as 6 hours after the intraperitoneal injection or the feeding of thiamine or cocarboxylase. They persist for at least 10 days. Choline, or lipocaic, injected simultaneously with the vitamin or the feeding of a diet high in casein failed to preserve or to restore the normal liver structure.

Histologically this pathologic change is characterized by a metamorphosis of the fat present in the liver cell. The severity of the structural changes depends upon the dose administered. 50 micrograms cause a slight histologic reaction, while 500 micrograms cause severe degeneration. This condition can be prevented in the rat by replacing the carbohydrate of the diet by fat. A normal liver structure is present in the fat-fed rats, although there is a slight increase in the fat content.

The Influence of Spermine and Spermidine on Tissue Oxidations.

By E. A. Evans, Jr. *From the Department of Biochemistry of the University of Chicago, Chicago*

The hyperglycemic action of dilute acid extracts of swine intestinal mucosa can be ascribed in part to the presence of spermine and spermidine, both of which were isolated from such extracts. There is little information concerning the physiological properties of these amines, although they occur in many mammalian tissues. Both when injected intramuscularly (15 mg. per kilo) into fasted rabbits evoke a hyperglycemia persisting for several hours. While this hyperglycemia can have many origins, the association of hyperglycemia with anoxia suggested the investigation of the effect of spermine on the respiration of isolated tissues. By means of the direct Warburg technique with minced guinea pig brain, the presence of 0.0057 M spermine was found to cause a 70 to 80 per cent inhibition of the oxygen consumption due to glucose. Similar results were obtained with spermidine. The extra oxygen consumption of minced brain tissue respiring on lactate or pyruvate was also inhibited by 0.0057 M spermine to about the same extent. The same concentration of spermine causes no inhibition with succinic acid as a substrate for brain, while only slight inhibition (10 to 20 per cent) occurs with glutamic acid. Little if any oxidation of spermine occurs in the presence of minced guinea pig liver, lung, or kidney. There is some evidence that oxidation of spermine occurs with minced brain but the reaction is slow and incomplete. While the data submitted do not furnish adequate proof for such a conclusion, it is possible that this inhibition is part of the normal function of spermine and spermidine.

On the Mechanism of Augmentation of Gonadotropic Activity.

By JOHN S. EVANS, JOSEPH CEITHAML, L. R. HINES, AND F. C. KOCH. *From Department of Biochemistry, the University of Chicago, Chicago*

The augmentation of gonadotropic activity of follicle-stimulating products prepared from pituitary tissue, by the addition of copper or zinc salts as observed by Pfeiffer, Fevold, Maxwell, and Saunders and Cole was confirmed by the van Dyke-Wallen-Lawrence method of assay.* Increasing amounts of copper sulfate up to a certain level with constant amounts of pituitary extract produce corresponding increases in ovarian weights. Adrenalin chloride (1:10,000) produces similar effects. No augmentation was observed when the mixtures of gonadotropic material and enhancing agent are injected directly into the heart. Only slight augmentations are observed when the gonadotropic material and enhancing agents are injected subcutaneously at different sites. Neither salmine nor $CaCl_2$ possesses the enhancing properties, even when injected with the gonadotropic substance. The striking action of adrenalin and the negative effects from cardiac injection support the view that the enhancing effects are due to delayed absorption. In contrast to pituitary tissue extracts, human pregnancy urine and pregnant mare serum (gonadogen, Upjohn) preparations were not augmented by copper sulfate.

---

* Pfeiffer, C. A., *Anat. Rec.*, **67**, 159 (1937). Fevold, H. L., Hisaw, F. L., and Grup, R., *Am. J. Physiol.*, **104**, 710 (1933); **117**, 68 (1936); *Endocrinology*, **21**, 343 (1937). Maxwell, L. C., *Am. J. Physiol.*, **110**, 459 (1934). Saunders, F. J., and Cole, H. H., *Endocrinology*, **23**, 302 (1938). van Dyke, H. B., and Wallen-Lawrence, Z., *J. Pharmacol. and Exp. Therap.*, **47**, 163 (1933).

The Determination of pH with the Photoelectric Colorimeter.

BY KENNETH A. EVELYN AND HELGA TAIT MALLOY. *From the University Clinic, Royal Victoria Hospital, Montreal, Canada*

The photoelectric colorimeter* has been applied to the determination of pH by means of indicators. The principle involved is essentially the same as that of the spectrophotometric determination, in that the color of the indicator is expressed in terms of the ratio of the optical densities of the solution measured at two different wave-lengths. The wave-lengths chosen for each indicator are isolated by selective light filters so as to correspond to the points of maximum absorption of the acid and alkaline forms of the indicator respectively.

The measurement is independent of subjective estimations of color, and does not involve the use of any standard buffer or indicator solutions. Although the photometric method is as rapid as the ordinary visual comparison procedure, the accuracy obtained is usually better than that of the spectrophotometric technique when even the best of visual spectrophotometers are employed. The pH of a buffer solution can be determined with a precision of better than 0.01 pH, but the absolute accuracy is dependent on the accuracy with which the results can be corrected for salt and protein errors. Subject to these reservations the method has been applied to the determination of the pH of plasma, by a modification of the Cullen procedure. The photoelectric method has also been found most satisfactory for measuring the pK of any indicator and for evaluating the effect on pK of various factors such as temperature and ionic strength.

---

* Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).

Thermostable Oxidations in Tumor Tissue.

By R. N. Feinstein and F. J. Stare. *From the Cancer Research Laboratories, University of Wisconsin School of Medicine, Madison*

The presence in muscle of a thermostable mechanism for oxidations and reductions dependent on glutathione was observed by Hopkins. At acid pH values he attributed the oxygen uptake of this system to fat oxidation, and at neutral or alkaline pH values to protein oxidation. We were interested in studying this system in rat carcinoma.

Thermostable powders were prepared from muscle and cancer tissue (Flexner-Jobling) by grinding, extracting with boiling water, washing with alcohol, and drying. Such powders suspended in phosphate slowly take up oxygen. Addition of a few mg. of glutathione increases the oxygen uptake 100 per cent or more. In the presence of a few mg. of glutathione, 1 gm. of muscle powder will consume 700 c.mm. of oxygen in 6 hours, after which oxygen is still being consumed at nearly the initial rate. The amount of oxygen is more than can be accounted for on the basis of the oxidation of the glutathione. The same amount of tumor powder prepared from either the healthy or the necrotic part of the tumor will consume only 180 c.mm. of oxygen. The oxygen uptake of fresh muscle, either slices or brei, is of the same order as the oxygen uptake of fresh tumor.

Apparently the thermostable oxidative system in which glutathione functions is markedly diminished (by 75 per cent) in tumor tissue (Flexner-Jobling rat carcinoma).

The Dielectric Properties of Lactoglobulin and Its Interaction with Ions and Dipolar Ions.

By J. D. Ferry, Edwin J. Cohn, J. L. Oncley, and Muriel H. Blanchard. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The significance of dielectric constant increments and of dipole moments of amino acids, peptides, and proteins for their interactions with ions and other dipolar ions has previously been stressed.*

Palmer† has measured the solubility of crystalline lactoglobulin in dilute sodium chloride solutions. Its interaction with salt is greater than that of hemoglobin or of any amino acid or peptide hitherto studied. It seemed, therefore, desirable to determine whether the dipole moment of this protein, and its interaction with dipolar ions, is also comparatively large.

The dielectric constant increment of lactoglobulin has been determined in aqueous solution and in the presence of 0.25 M and 0.50 M glycine to be 1.4 per gm. per liter, the largest of any proteins thus far studied; or 56,000 per mole, on the basis of a molecular weight of 40,000. The dipole moment is estimated to be 700 Debye units.

Paralleling the large influence of salt upon the solubility is the influence of the dipolar ion glycine. The solubility of lactoglobulin in glycine solutions is given by log $N/N_0 = KC$, where N_0 is the solubility in water and N that in a solution of glycine of concentration C. The constant K has the value 2.0, as compared with 0.10 for asparagine, 0.23 for cystine, and 0.95 for hemoglobin, in glycine solutions.

Comparison with dipole moments (15 for asparagine, 27 for cystine, and 500 for hemoglobin) indicates the importance of the moments in these interactions.

Studies on the Proteins of the Kidney. I. By Ronald M. Ferry, Virginia T. Ferry, Jr., and Mary K. Connolly. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The possibility that protein structure, already shown to be directly connected with the function of muscle, might be definitely related to the activity of other tissues induced us to undertake a study of the proteins in the renal cortex.

Horse kidneys were removed immediately after death, perfused free of blood with physiological saline, and the cortex dissected and immediately frozen. The frozen tissue was finely ground and either kept at −10° or −15° or dried rapidly in a vacuum.

---

* Cohn, E. J., *Chem. Rev.*, **19**, 241 (1936); *J. Physic. Chem.*, **43**, 169 (1939).

† Palmer, A. H., *J. Biol. Chem.*, **104**, 359 (1934).

Extracts were made from these materials with salt solutions varying in the kind of salt, pH, and ionic strength. At ionic strengths between 0.5 and 1.0, no significant variation in protein concentration was noted. Higher concentrations were less effective. The extracts in sodium and potassium chlorides and especially in phosphate buffers seemed more stable. Variation in pH from 6.2 to 7.4 showed no appreciable change in the concentration of protein extracted at constant ionic strength, although the character of the solution did vary. Within the limits given, the concentration of extracted protein was strikingly constant at 0.5 to 0.6 per cent and represented 28 to 32 per cent of the dry weight of the cortex. About 60 per cent of this is globulin.

The more alkaline phosphate buffer extracts from fresh tissue were more viscous and were thread-forming, suggesting those from muscle. Unlike myosin solutions, they do not show double refraction of flow. The substance giving rise to this property has the characteristics of a mucin.

Carbohydrate Changes in Working Muscle. By Eunice V. Flock and Jesse L. Bollman. *From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*

When muscles of rats are stimulated to contract isotonically *in vivo* at the rate of three twitches per second for varying time intervals, it is found that muscle glycogen, which breaks down rapidly, reaches a low level at the end of the 1st minute of contraction and remains approximately at this level as work continues. Associated with the glycogen breakdown during the 1st minute of work are marked increases in lactic acid and hexosephosphate and much smaller but definite increases in pyruvic acid and glucose. The concentration of glucose may remain elevated during subsequent work, whereas the concentrations of lactate, pyruvate, and hexosephosphate decrease to normal values within 15 or 20 minutes and remain normal during more prolonged work. No difference is found in the rate of disappearance of these three substances, whether the muscle works or rests after the 1st minute of contraction.

The finding of an increase in pyruvic acid in the muscle during the initial stages of work suggests that the increased pyruvate in blood and urine in the human after severe exercise, observed by Johnson and Edwards, is of muscular origin. Rats in a state of vitamin B_1 deficiency do not differ from normal rats in the magnitude or duration of increase of pyruvate with work, although such rats have a higher initial concentration of pyruvate in both blood and muscles.

Gastric Emptying of Cereal Starch. BY PAUL F. FROESCHLE AND H. B. PIERCE. *From the Department of Physiological Chemistry, College of Medicine, University of Vermont, Burlington*

Cereal starch has been fed at two levels to 130 young adult female rats fasted for 48 hours. The animals were sacrificed at definite time intervals after having food placed before them, and unabsorbed cereal was recovered from the stomach and intestine. The amount of starch and free sugar was determined in the contents of the stomach and small intestine. Knowing the amounts of carbohydrate fed and recovered and seat of recovery, we have computed the rates of gastric emptying and absorption.

At the two levels fed, approximately 170 and 280 mg. of dry cereal starch, the weight of material leaving the stomach was less with the small than with the large amount, but the percentage of the total leaving the stomach was approximately the same in the two series. This would afford less carbohydrate for absorption when the small quantity was fed. The total weight of carbohydrate absorbed, in turn, was found to be lower with the lower intake, whereas the percentage of the total absorbed for each group was approximately the same.

Curves plotted showing the amount of starch hydrolyzed and amount of carbohydrate absorbed indicate that hydrolysis proceeds at a more rapid rate than absorption in early, but not late periods of the experiment.

The Rôle of Iron, Copper, and Cobalt in Hemoglobin Production in Dogs on a Milk Diet. BY D. V. FROST AND C. A. ELVEHJEM. *From the Department of Biochemistry, University of Wisconsin, Madison*

A severe copper deficiency was produced in young dogs by feeding anemic dogs 20 mg. of Fe per day for 1 to 3 weeks to deplete the copper reserves and returning the dogs to milk alone until hemoglobin values of 5 to 7 gm. per 100 cc. of blood were obtained. Addition of 20 mg. of Fe at this point produced no significant increase in hemoglobin, but the addition of 20 mg. of Fe and 2 mg. of Cu produced rapid increases. When 10 mg. of Fe and 2 mg. of Cu were fed, 50 per cent of the ingested iron was used directly for hemoglobin production during the first 3 weeks of therapy.

Several of the dogs that had been made anemic by the above method and kept on a milk diet for 3 to 5 months showed an excellent original response to iron and copper but the hemoglobin level reached a plateau at 10 to 12 gm. for 100 cc. of blood. As little as 0.1 mg. of Co per day stimulated Hb production in these dogs and allowed complete regeneration.

Dogs maintained on milk and made anemic by hemorrhage attained normal hemoglobin levels rapidly on dry whole liver and on iron plus copper and slowly and incompletely on iron alone.

When dogs made anemic by hemorrhage were given 4 mg. of cobalt per day followed in 2 weeks by iron and copper, they failed to show a hemoglobin or weight response. Addition of whole liver promoted a rapid increase of hemoglobin. The calculated hemoglobin increase during the 2nd week of liver therapy was as high as 76 gm. Later polycythemia developed.

Effects of an Anterior Pituitary Growth Preparation on Metabolism in Phlorhizin Diabetes.

By Oliver Henry Gaebler and Walter J. Zimmerman. *From the Department of Laboratories, Henry Ford Hospital, Detroit*

In previous experiments on normal dogs protein catabolism was greatly decreased by injections of a growth preparation, despite a large increase in the basal metabolic rate. This state of affairs was accounted for by increased oxidation of fat or carbohydrate, depending upon the dietary régime. We have now studied the question of whether oxidation of carbohydrate is essential in this process, or whether protein catabolism can also be reduced if fat alone supplies the remaining calories. We were also interested in knowing whether the growth preparation altered the yield of glucose from protein.

Normal, phlorhizinized, and phlorhizinized thyroidectomized dogs were used. They received a constant quantity of meat daily. In the normal dogs the increased oxidation of fat caused by the growth preparation was unaccompanied by ketosis. In the phlorhizinized dogs the growth preparation increased still further the high metabolism of phlorhizin diabetes and correspondingly augmented the ketosis. In spite of these untoward effects the urine nitrogen decreased slightly for 1 day in some of the animals, the non-protein nitrogen of the blood remaining constant. In the thyroidectomized phlorhizinized dogs the nitrogen storage produced by the growth preparation was more pronounced, though still much smaller than in normal animals. The effectiveness of the growth preparation was greatly diminished, but not abolished by phlorhizin. The familiar D:N ratio was unaffected by the growth preparation in either group of phlorhizinized animals, so that the yield of glucose from protein catabolized appears to remain unchanged.

Renal Threshold for Glucose in Pregnancy. By R. B. Gibson, Joyce Findlayson, and W. D. Paul. *From the Pathological Chemistry Laboratory, University Hospital, State University of Iowa, Iowa City*

Observations were made during late pregnancy on the renal threshold for glucose. Sherrill and MacKay's* procedure, with certain modifications, was followed. After fasting 12 hours, the patients received 200 cc. of water at 6 a. m. and 200 cc. each half hour thereafter. At 7.30 a. m., they were given 0.25 gm. of urea per kilo of body weight; at 8 a. m. 10 units of insulin. A retention catheter was introduced. Urines were collected over 20 minute periods and blood drawn from the finger tip in the mid-periods. At the conclusion of a control period, 25 gm. of glucose in 50 per cent solution were administered intravenously. Urine sugar was fermented with yeast (or *B. coli communior*) and the urine treated with acid-washed Lloyd's reagent. Blood sugars for the periods preceding minimum and constant content of urine fermentable sugar were considered threshold values. Thresholds found for the descending curves (twelve women) averaged 78 mg. per cent, from 40 to 152 mg. per cent; two non-pregnant women showed 102+ and 152 mg. per cent. Sherrill and MacKay found average thresholds of 128 (78 to 157) mg. per cent in eleven normals. Thresholds obtained for five of our patients during the recovery phase exceeded those of the descending blood sugars (averaging for descent 60 mg. per cent; for recovery, 73 mg. per cent). The results demonstrate definite lowering of the renal threshold in late pregnancy—explaining the non-diabetic glycosuria often encountered—and are in accord with earlier findings where other techniques were employed.

---

* Sherrill, J. W., and MacKay, E. M., *Arch. Int. Med.*, **58**, 877 (1935).

Specificity of Choline Esterase. By DAVID GLICK. *From the Research Laboratories of the Newark Beth Israel Hospital, Newark*

Thioacetyl esters of choline are enzymatically hydrolyzed much faster than the corresponding oxygen compounds. Introduction of a halogen into the acid component of acetylcholine also makes the compound more susceptible to enzyme action. Substitution of methyl, ethyl, phenyl, or piperidinoyl groups into the acid component of carbaminoylcholine has no effect upon the resistance to enzyme action possessed by the ester. The piperidinium and pyrrolidinium analogues of acetylcholine (N-dimethyl-α-acetoxymethylpiperidinium iodide and N-dimethyl-2-acetoxymethylpyrrolidinium iodide) are both split 60 per cent as rapidly as acetylcholine itself. Only slight enzyme activity is observed with the piperidinium analogue of acetyl-γ-homocholine, and none when a β-methyl group is placed in the piperidinium component. β-Alkyl substitutions, with and without alkyl changes on the nitrogen, in the pyrrolidinium analogue of acetylcholine show no enzyme action. Employing the pyrrolidinium group as the acid component of an ester, analogous to the betaine type esters, rendered the compounds impervious to enzymatic hydrolysis. From these data, and others previously reported, a formulation may now be made of certain general rules concerning the relation between structure and enzymatic scission of choline and choline-like esters.

The Capsular Polysaccharide of Type XIV Pneumococcus and Its Relationship to the Specific Substances of Human Blood. By WALTHER F. GOEBEL, PAUL B. BEESON, AND CHARLES L.

HOAGLAND. *From the Hospital of The Rockefeller Institute for Medical Research, New York*

Since Finland and Curnen* have shown that Type XIV antipneumococcus horse serum contains agglutinins for human erythrocytes of different blood groups, a study has been made of the Type XIV specific polysaccharide and its relationship to the chemical and immunological properties of the blood group specific substances. The capsular polysaccharide isolated from Type XIV pneumococci is a nitrogenous carbohydrate constituted from molecules of an acetylated amino sugar and a hexose. The bacterial carbohydrate resembles in chemical and immunological properties the blood Group A specific substance, the only blood group specific substance whose properties are well established.

Type XIV antipneumococcus horse serum precipitates the blood Group A specific substance in high dilution. When Type XIV antipneumococcus serum is absorbed with the bacterial carbohydrate, not only are all homologous precipitins and agglutinins removed, but agglutination of human erythrocytes and precipitation of the blood Group A specific substance no longer take place. Absorption of the serum with the A substance or with erythrocytes of A, B, or O groups removes the agglutinins for all blood groups, but not the antibodies which react with Type XIV pneumococci or with the capsular polysaccharide derived from them.

Others have reported that administration of Type XIV antipneumococcus horse serum to pneumonia patients has in some instances been followed by hemoglobinuria and death. These untoward reactions can be attributed to a similarity in structure of the Type XIV specific substance and the blood group polysaccharides, as reflected in the immune serum.

Sulfhydryl Groups in Proteins. BY JESSE P. GREENSTEIN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

Egg albumin, edestin, excelsin, and globin, when in the native, unaltered state do not give tests for sulfhydryl groups. When, however, these proteins are treated in any one of several ways, such as by heat, shaking, acetone precipitation, or by solution in urea, free sulfhydryl groups appear.

---

* Finland, M., and Curnen, E. C., *Science*, **87**, 417 (1938).

The number of —SH groups which appear in these proteins in the presence of urea, guanidine hydrochloride, and some of their derivatives has been estimated by the use of the porphyrindin dye. Certain of the results may be summarized as follows: (1) the proportion of —SH groups appearing in either urea or guanidine is much greater than when the proteins are treated by heat, shaking, or precipitation; (2) guanidine hydrochloride has the greatest effect observed thus far in liberating —SH groups; (3) the proportion of —SH groups appears to be independent of the protein concentration, and in the case of egg albumin is dependent only on the concentration of urea or guanidine hydrochloride; (4) in equal concentrations of guanidine hydrochloride, the —SH groups determined amount in egg albumin to two-thirds, in edestin to one-third, in excelsin to one-ninth, and in globin to three-fourths of the alkali-labile sulfur in each protein; (5) there is no apparent relation between the differences in molecular weight of certain proteins in water and urea and the amount of —SH groups which appear in the latter solvent.

The Relation of Growth, Dietary Protein, and Liver Fat in Young Rats. By Wendell H. Griffith. *From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

The rate of growth of young rats and the production of fatty livers have been determined on high fat diets, with and without added choline and with and without adequate protein. Male rats weighing 40 gm. were used in groups of ten and were continued on the experimental diets for 28 to 30 days. The vitamin supplement was constant and consisted of dried liver, yeast, and cod liver oil. Casein, fibrin, and egg albumin, alone and in various combinations, were tested at 5, 10, and 15 per cent levels. In general a direct relationship was found between the rate of growth and the amount of liver fat. One of the better rates of growth (2 gm. daily for 30 days) associated with a high liver fat (35 per cent) resulted from the feeding of the following mixture: casein 4, fibrin 4, egg albumin 2, yeast 5, liver 2, salt mixture 5,* agar 2, cod liver oil 5, sucrose 36, and lard 35. In every case the addition of choline prevented the increase in liver fat. No evidence was obtained that choline possessed growth activity if the dietary protein was adequate.

---

* Hawk and Oser mixture 4, calcium carbonate 1. (Hawk, P. B., and Oser, B. L., *Science*, 74, 369 (1931).)

The Carbohydrate of the Gonadotropic Hormone of Pregnancy Urine. By Samuel Gurin, C. Bachman, and D. Wright Wilson. *From the Departments of Physiological Chemistry and Gynecology and Obstetrics, University of Pennsylvania, Philadelphia*

Analyses of the carbohydrate in the gonadotropic hormone of pregnancy urine by various methods have been made. The following values were obtained for several highly purified preparations: Shaffer-Hartmann method, 17 to 19 per cent; Shaffer-Hartmann method after use of $Zn(OH)_2$, 13 to 16 per cent; carbazole method, 9 to 11 per cent; orcinol method, 9 to 11 per cent; all calculated as galactose. Hexosamine varied from 4.5 to 6 per cent and the molecular ratio of galactose to hexosamine was 2.10 ± 0.13. The carbazole method was studied in detail and found to be superior to the orcinol method for identifying the sugar in a carbohydrate-protein complex such as the hormone. Data obtained with the carbazole method, after certain necessary corrections were applied, demonstrated that the carbohydrate of the gonadotropic hormone is galactose. Confirmatory data were obtained by using the orcinol method.

A Property of the Hydroxy Group in Amino Acids. By Frederick S. Hammett. *From the Lankenau Hospital Research Institute, Philadelphia*

A systematic survey of the part played in growth by the naturally occurring amino acids has brought out hitherto unsuspected properties. Thus it has been found that there is a consistent difference in type in the three OH-bearing compounds serine, tyrosine, and hydroxyproline, and their non-OH-bearing congeners alanine, phenylalanine, and proline. The introduction of the OH group results in a lessened toxicity, a lessened specific dynamic effect, and in bringing to the fore a characterizing growth influence. The observations were all made on the colonial-living form of the marine hydroid *Obelia geniculata.*

An Oil Manometer for the Fifteen-Fold Increase in Sensitivity of the Manometric Gas Apparatus of Van Slyke and Neill. By Martin E. Hanke. *From the Department of Biochemistry, the University of Chicago, Chicago*

The apparatus consists of a pair of kerosene manometers, 2 mm. bore, above mercury reservoirs, 25 mm. bore, joined below through a U-bend to the mercury of the regular gas apparatus by a side arm just above the leveling bulb stop-cock. The oil manometers are at about atmospheric pressure, and the position of the 25 mm. bore tube containing the mercury-oil meniscus is about 76 cm. below the reaction chamber. The manometer readings are made independent of atmospheric pressure changes by joining their upper ends to a gas reservoir (4 liter bottle) whose pressure measured on a separate oil barometer is adjusted to a definite value by inflow or outflow of water immediately before each manometric reading. Stop-cocks allow one to cut out either one or both oil manometers, and the regular usefulness of the apparatus with the mercury manometer remains unaltered. Temperature of the chamber is read to 0.01° to correct for aqueous tension changes. The reaction chamber is rigidly braced during the manometric readings so as to make its position uniform to 0.01 mm.

For the precise analysis of small samples, small and uniform blanks are realized by the use of gas-free reagents and precise volume measurement of reagents. In the determination of CO_2, O_2, urea, and non-protein N of blood, blanks constant to 0.6 mm. of oil have been obtained, and accuracy of 1 part in 200 on 0.1 cc. samples of blood (1 micromole of gas) has been realized.

An Aerometric Method for the Rapid Determination of Alcohol in Water and Body Fluids. By R. N. Harger, E. G. Bridwell, and B. B. Raney. *From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis*

Air is brought to equilibrium with the fluid containing alcohol and then analyzed for alcohol content. From this figure, and the partition ratio of alcohol between air and the water phase for this temperature, one can calculate the concentration of alcohol in the water phase.

Alcohol equilibrium is obtained by forcing the air once through a 2 inch layer of the fluid by means of a fritted glass gas-distributing disk. The concentration of alcohol in the air phase is determined by passing this air through our sulfuric acid-permanganate reagent.* The volume necessary to obtain the end-point represents a constant weight of alcohol. No thermostat is needed, as the fluid temperature is near that of the room and the entire operation requires only about 2 minutes. The fluid temperature must be recorded with an accuracy of about 0.1°, since the partition ratio of alcohol between the two phases changes about 6 per cent for every degree.

Since the published data on the partition ratio of alcohol between air and water are fragmentary and somewhat conflicting, we have determined this ratio for temperature intervals of 5° ranging from 0–40°. That these figures represent equilibrium was shown by employing air with initial alcohol concentrations above, and also below, equilibrium values. Similar data were obtained for urine and blood.

Analyses of unknowns showed an average error of ±2.4 per cent for water solutions and ±4.1 per cent for urine solutions of alcohol.

Evidence of Hyperfunction of the Anterior Pituitary in a Strain of Rats. By Ben K. Harned and Versa V. Cole. *From the Laboratory of Pharmacology, Woman's Medical College of Pennsylvania, Philadelphia*

With glucose tests, water consumption and urine excretion per gm. of body weight, and rate of growth as criteria, the data demonstrate a marked difference between two strains of rats grown in our laboratory on the same diet and under the same conditions for four to eight generations.

The stock for Colony A was obtained from the Laboratory of Physiological Chemistry at Yale but owing to our breeding policy the present colony, possibly, is sufficiently different from the parent colony to justify the designation, Woman's Medical College strain. The stock for Colony B was obtained from the Wistar Institute and has retained the characteristics of the parent colony.

Previous reports have shown that a high percentage of the animals from Colony A give glucose tolerance curves diabetic in type and recent data indicate that the low tolerance is an inherited characteristic.

---

* Harger, R. N., Lamb, E. B., and Hulpieu, H. R., *J. Am. Med. Assn.*, **110**, 779 (1938).

Studies of the water consumption and urine excretion of 94 unselected rats, paired according to age, from the two colonies demonstrate a significantly higher volume of water consumed and urine excreted per gm. of body weight by animals from Colony A. The urine is sugar-free.

Growth curves show that the average weaning weight of Colony A is 23 per cent greater than that of Colony B and that at 200 days the difference has increased to 42 per cent. Per cm. of body length (nose to anus) the average weight of Colony A exceeds that of Colony B by 20 per cent.

The Effect of Vitamin A and Carotene on Goiter Due to Low Iodine Intake.

By Philip L. Harris and Roe E. Remington. *From the Food Research Laboratory, Department of Chemistry, Medical College of South Carolina, Charleston*

By the technique developed in this laboratory, wherein the degree of hyperplasia induced by an iodine-deficient régime is quantitatively established by the measurement of thyroid weight in relation to body weight, of the percentage of dry matter, and of the iodine content of the thyroid, a study was made of vitamin A-iodine metabolism relationships. A rather extensive literature exists concerning this subject but the results are, in general, inconclusive and contradictory.

Young goitrous rats fed a vitamin A-deficient diet developed typical symptoms of avitaminosis as soon as did litter mates which were receiving the same diet supplemented with the minimal amount of iodine to prevent goiter. Furthermore, the survival time of goitrous rats was no different from that of rats with normal thyroids when the vitamin A-free diet was continued until death.

The level of vitamin A or of carotene intake has no appreciable effect on the production or severity of goiter due to low iodine intake. Rats, depleted of vitamin A, were fed 3, 30, and 300 I.U. of vitamin A *per se* (product of molecular distillation) or as β-carotene and no differences in the severity of the goiter produced under these conditions could be demonstrated. This same experiment was repeated except that the animals received enough iodine just to prevent goiter. The results show that neither vitamin A nor carotene has any influence on the utilization of minimal amounts of iodine.

Hydrolysis of the Conjugated Estrogens of Pregnant Mare Urine.

By R. D. H. Heard and M. Edson. *From the Connaught Laboratories, University of Toronto, Toronto, Canada*

In order to estimate the total female sex hormone content of urine, quantitative conversion of the relatively inactive water-soluble conjugated estrogens into the physiologically potent ether-soluble free hormones is first necessary. While optimum hydrolysis of the combined forms in human urine is effected by autoclaving at pH 1 to 2 for 2 to 4 hours, the application of heat to acidified mare urine results in considerable destruction of the liberated estrin. The rate and extent of the hydrolysis at room temperature of the conjugated estrogens excreted by the mare during early, middle, and late pregnancy were therefore ascertained under different conditions of hydrogen ion concentration.

Maximum conversion was obtained by allowing urine acidified to pH 0.4 to 0.6 (quinhydrone electrode) to stand for at least 4 weeks. The amount of released estrin increased at an approximately linear rate during this period and remained practically constant throughout the succeeding 3 weeks. Below pH 0.4, evidence of destruction of an appreciable quantity of the product was observed. Above pH 0.6, hydrolysis was less complete; at pH 1.0, the extent of the conversion was only 60 to 75 per cent of that at pH 0.4 to 0.6. The same optimum conditions apply throughout gestation, regardless of the known variations in the relative amounts of the different estrogens excreted.

Gonadotropic Hormone: Assays of Human Male Urine.

By Emily J. Heller, Carl G. Heller, and Elmer L. Sevringhaus. *From the Department of Medicine, University of Wisconsin, Madison*

By a modification of the Zondek alcohol precipitation method for gonadotropic substance, 12 hour night specimens of urine were extracted and assayed by the immature rat uterus method. A series of forty normal men ranging in age from 20 years to 85 years has been studied. From 100 cc. to 400 cc. equivalents of the original urine were necessary to cause uterine weight increase in a rat. Gonadotropic activity was found in the urines of all normal male subjects. No consistent increase in potency with increase in age could be demonstrated. When assays of male urine are compared with similar assays performed on urines from normal women and from women during menopause, several striking differences between the excretion of gonadotropic hormones in the male and in the female become apparent. (*a*) Urinary excretion by the males is more variable. (*b*) The normal male excretes gonadotropic hormone at a higher level than the normal female except during the intermenstrual rise. (*c*) The rapid rise and great increase in potency of the urine in the female at the menopause is not duplicated in the male with advance in years. (*d*) At no age period do the males excrete amounts of hormone comparable to that found in menopausal or postmenopausal women. Men with involutional symptoms tend to show higher urinary potency than normal males.

Purification of Renin. By O. M. Helmer and Irvine H. Page. *From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis*

In view of the renewed interest in renin as the possible effective substance responsible for hypertension of renal ischemic origin, it seemed desirable to attempt purification of the relatively impure extracts which have been employed for studying the physiological properties of the pressor substance.

Finely ground pig kidney cortex is extracted with acetone, washed with ether, dried, and powdered. A NaCl solution of the powder is freed from the bulk of the inactive protein by an isoelectric precipitation. Another inactive protein fraction is removed at 1 M potassium phosphate concentration at pH 6.5. The active pressor substance is precipitated between 1 M and 2 M potassium phosphate concentration at pH 6.5. This highly active fraction is further purified by precipitating 90 per cent of the protein nitrogen and practically all of the pigment by adjusting to a concentration of 5 per cent acetic acid and 5 per cent NaCl and dialyzing against distilled water. From this water-clear solution strongly pressor reineckates and picrates can be prepared. Ammonium sulfate concentration of 40 per cent at pH 3 to 4 and sodium chloride at full saturation at the same pH will also precipitate active pressor substance. The latter material will cause a rise in blood pressure of 30 mm. or more in cats in quantities of 0.009 mg. of nitrogen per kilo of body weight. Renin has the physical and chemical properties of a protein.

The Acid Sensitivity of Cystine Peptides and Anhydrides. By W. C. Hess and M. X. Sullivan. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

In work with crystalline insulin it was found that certain samples of insulin yielded smaller amounts of cystine when hydrolyzed with HCl alone than when hydrolyzed with a mixture of HCl and HCOOH.* The cystine content of other proteins showed practically no difference between the two types of hydrolysis. This suggested the possibility that the cystine linkage in insulin was of a different character than in other proteins. Accordingly various cystine peptides such as diglycylcystine, cystinyldiglycine, cystinylcystine, and cystinyldiglutamic acid were hydrolyzed with both HCl and HCl-HCOOH. Both types of hydrolysis yielded the theoretical amount of cystine. On the other hand the anhydride of cystinyldiglycine gave a lower value with the HCl hydrolysis than it did with the HCl-HCOOH. The work is being continued with other cystine derivatives and anhydrides thereof.

The Nature of Vitamin A in Fish Liver Oils, and the Need for a New International Standard. By K. Hickman. *From the Distillation Products Corporation, Rochester, New York*

In attempting to fix a standard factor for converting extinction coefficients of vitamin A preparations into biological units, it has hitherto been assumed that all such preparations, if adequately assayed, would show the same factor. The authors now find that the esters of vitamin A have a different factor from the free vitamin and that each preparation of vitamin A has a characteristic factor which depends on the state of combination of the vitamin, the nature of the solvent, the presence of anti- or synergistic substances, and the kind of diet on which the test animals were depleted. The evidence, which does not preclude the possibility of other growth-promoting substances in addition to vitamin A, is gathered chiefly from experiments in molecular distillation. The distilled esters have a factor as great as the vitamin in the crude oils; saponified concentrates always have a smaller factor, which sometimes may be less than half. Liver oils from the cod family have a higher factor of international units to ester than oils from most other species.

---

* Miller, G. L., and du Vigneaud, V., *J. Biol. Chem.*, **118**, 101 (1937).

The Cause of the Fall in Body Temperature of "Immobilized" Rats Exposed to Low Environmental Temperatures.

BY ROBERT M. HILL AND FRED SCHULTZ. *From the Department of Biochemistry, University of Colorado Medical School and Hospitals, Denver*

Hamilton observed that, when rats are closely confined, the body temperature falls on exposure of the animals to low environmental temperatures. He believed this fall to be an effect of immobilization.

In our experiments rats, exposed in the cold room (2–6°) in small individual wire cages, were quiet and showed no tendency to lowered body temperatures unless the cage was tight enough to impede the animal's breathing. In the tight cages, where the animals usually struggled, there was a rapid fall in body temperature to 15–20°.

These experiments were repeated with animals "immobilized" by being strapped to wooden splints with adhesive tape. "Immobilized" animals with the breathing impeded as little as possible showed falls in body temperature of 3–5° in 4 to 6 hours. When a single 1 inch tape was placed about the thorax, the body temperature fell to 15–20°, sometimes in less than 1 hour. The animals without the thorax strap were much more quiet and showed much less fall in body temperature than those with the thorax strap.

Gellhorn has shown that decreased O_2 tension and increased CO_2 tension in the inspired air will cause a lowered body temperature in rats exposed to low environmental temperatures. We believe that lowered O_2 tension and increased CO_2 tension in the tissues due to interference with breathing is the cause of the failure of our animals to maintain normal body temperatures when exposed to a cold environment and that immobilization is not a factor.

The Hydrolysis of Adenosinetriphosphate by Bone and Venom Phosphatases. BY GEORGE H. HITCHINGS AND HENRY S. FULLER. *From the School of Public Health, Harvard University, Boston*

Adenosinetriphosphate is hydrolyzed by bone phosphatase into equimolar quantities of adenosine, pyrophosphate, and inorganic phosphate, and not, as Barrenscheen* supposed, into adenosinediphosphate and orthophosphate. Venoms from snakes of the genus *Crotalus* catalyze the same reaction, but show relatively greater activity. This enzymatic reaction has been studied in some detail. The significance of the findings as related to the structure of polyphosphonucleotides will be discussed.

Solubility Studies on the Calcium Phosphates. BY HAROLD CARPENTER HODGE. *From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

Titrations at 37° were carried out by the addition of $Ca(OH)_2$ to H_3PO_4 and *vice versa*, with amounts of each sufficient to space the systems along the titration curve from pH 6 to 11. The systems were shaken at 37° for about 6 weeks, and precipitates and filtrates obtained and analyzed chemically and by x-ray diffraction patterns. The final pH depended, for a given initial pH, on the amount of precipitate present. Evidence was obtained for the existence of a portion of the $CaO-P_2O_5-H_2O$ system arising from hydroxyapatite. Solubility products were calculated. The effect of mode of precipitation appears in the phase diagram in a manner consonant with previous theories concerning the nature of hydroxyapatite precipitates.

Further Studies of the Posthemolytic Residue of Erythrocytes. On the Chemical Composition of the Stroma of Erythrocytes in Pathological Conditions, with Special Attention to Polycythemia in Man. BY OLIVE D. HOFFMAN AND ELIOT F. BEACH. *From the Research Laboratory of the Children's Fund of Michigan, Detroit*

Posthemolytic residues have been prepared according to the previously published method from the erythrocytes (1) of humans with polycythemia, (2) of dogs with N-propyl disulfide anemia, (3) of horses donating tetanus antitoxin, and (4) of horses donating diphtheria antitoxin.

---

* Barrenscheen, H. K., and Jachimowicz, T., *Biochem Z.*, **292**, 350 (1937).

The protein preparations after lipid extraction were analyzed according to the method of Block for the basic amino acids and by the method of Folin and Ciocalteu for tyrosine and tryptophane.

In polycythemia cases (controlled by bleeding) the per cent composition of arginine (4.21 to 4.79), lysine (3.26 to 3.91), tyrosine (2.54 to 2.98), and tryptophane (1.12 to 1.31) does not vary greatly from the normal, but tends to be low. Histidine was found to be low in preparations containing smaller amounts of total nitrogen. Protein represents approximately 71 per cent of the posthemolytic residue and lipid values account for 18.3 per cent. Of the total nitrogen, arginine accounts for approximately 12 per cent, histidine for 3 per cent, lysine for 5 per cent, tyrosine for less than 2 per cent, and tryptophane over 1 per cent. Thus approximately 23 per cent of the nitrogen has been accounted for.

Amino acid values of posthemolytic residue of anemic dogs were normal with the exception of histidine content, which was increased. All lipid fractions studied were markedly decreased except sphingomyelin. Erythrocyte stroma of horses donating tetanus and diphtheria antitoxin gave approximately normal amino acid values, although there was a decrease in arginine.

The lipid and mineral distribution relationships of the bloods will be discussed.

Anemia in Chicks Due to Vitamin Deficiency. By Albert G. Hogan and E. M. Parrott. *From the Laboratory of Agricultural Chemistry, University of Missouri, Columbia*

Chicks have been reared, with a variable degree of success, on simplified rations which contained the following vitamin carriers: cod liver oil 2, wheat germ oil 4, acid-hydrolyzed yeast 4, ether extract of egg yolk 2, tikitiki 1, water extract of liver 4. The numerals are parts per 100. When an alcohol extract of liver was substituted for the water extract the growth rate was subnormal and in 4 to 5 weeks the chicks became anemic. In recent studies the amount of the liver extract has been changed to 2 per cent, because when the quantity is reduced the anemia develops in a shorter time and more uniformly. If curative measures are not applied, the red cell counts continue to fall and the mortality is 100 per cent. The anemia is of the macrocytic, hyperchromic type, and the erythrocytes have increased resistance to hemolysis in hypotonic sodium chloride solution. The coagulation time of the blood is normal. The failure to maintain a normal red cell count is not caused by a subnormal food consumption. It is due to a vitamin deficiency, but the evidence indicates that it is not prevented or cured by any known vitamin previously described.

Errors in the Dilution Indicator Technique of Gastric Analysis.*

By Franklin Hollander and Joseph Bandes. *From the Laboratories of the Mount Sinai Hospital, New York*

In the dilution indicator technique for gastric analysis, some substance capable of quantitative determination is included in the test meal for the purpose of determining the proportions of test meal and mixed digestive secretion in a sample of gastric contents. By means of these proportions, the observed acid and chloride concentrations can be corrected to yield the corresponding values for the undiluted secretion. With phenol red as indicator in an alcohol test meal on three dogs which had not been operated upon, it was observed that the corrected chloride concentrations were often considerably greater than 165 mM (the value corresponding to an approximately isotonic solution of NaCl) and sometimes more than twice this value. Acidities behaved similarly, but much less frequently. These widely aberrant results may be ascribed to (1) the occurrence of an excessively hypertonic secretion, which is improbable, or (2) gastric absorption of a small volume of water and alcohol, with arithmetical magnification of its influence by the procedure employed in calculating the corrected concentrations. In experiments with isotonic saline and phenol red as a test meal, with correction for its own chloride content, such excessively hypertonic values were almost never observed; this supports the second explanation. Smaller fluctuations of the corrected chloride concentrations around 165 mM are ascribed in part to the admixture of non-acid secretion of lower chloride concentration and in part to a second source of error, which arises by arithmetical magnification of the small error in the phenol red determination. Failure to consider these sources of error in experiments like the foregoing may lead to erroneous interpretations.

---

* Supported in part by a grant from the Friedsam Foundation.

The Progressive Attenuation of Insulin by Interfacial Adsorption.

By J. M. Johlin. *From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville*

When aqueous solutions of crystalline insulin are subjected to the catalytic action of surface concentration and orientation by emulsification with chloroform for a period of 6 to 8 hours, practically all of the insulin is converted into an insoluble birefringent product whose physiological behavior is similar to that of other insoluble insulin products, such as protamine insulin. This modified form of insulin remains insoluble at a pH around 2.5, at which insulin solutions are usually prepared. Insulin, similarly treated for a period of 4 hours or less, exhibits the physical properties and physiological behavior of untreated insulin; when treated for longer periods of time than 8 hours, its insulin activity is gradually diminished as the time of treatment grows longer. Commercial insulin is not as readily attenuated as crystalline insulin. Data were obtained on the basis of several hundred individual experiments in which various insulin fractions were injected into standardized rabbits and blood sugar determinations made at repeated intervals following. The insulin distribution among different fractions was determined by nitrogen determinations. Insulin solutions used for treatment were prepared at pH 2.2 to 2.5 with saline which was either acidified with HCl or adjusted with 0.02 M McIlvaine's buffer.

Lactic Acid As a Component of the Physicochemical System of Human Blood.

By R. E. Johnson and J. W. Wilson. *From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston*

Lactic acid in the blood may become a quantitatively important component after severe exercise. When blood is equilibrated and analyzed by the techniques of L. J. Henderson and his associates, and lactic acid is estimated as well as the usual eight components (H_2O, HCl, cell protein, plasma protein, cell base, plasma base, CO_2, and O_2), lactic acid conforms to the "law of the blood" as formulated by Henderson. The Donnan ratios for chloride and lactate are of the same order of magnitude, and show similar changes with changing pO_2 and pCO_2.

If the rates of diffusion of lactate and chloride from plasma into red cells are studied at different temperatures by mixing plasma of high lactate or chloride content with red cells of low lactate or chloride content, and by drawing off samples at intervals, the rate of diffusion of chloride is too high to be measured by this technique, but the rate of diffusion of lactate is slow enough to be measured. At 0° almost no lactate enters the cells in an hour, and several minutes are required for attaining an equilibrium at 37°. *In vivo* the rate of diffusion is approximately the same as it is in freshly drawn blood *in vitro* at the same temperature. This can be demonstrated by drawing blood samples at short intervals after a brief bout of severe exercise.

The Effect of Storage on the Proteins of Seeds and Their Flours. Soy Beans and Wheat. BY D. BREESE JONES AND CHARLES E. F. GERSDORFF. *From the Protein and Nutrition Research Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*

Marked changes occur in the proteins of seeds on storage or aging, the extent varying with the kind of seed and conditions of storage. Although occurring much more rapidly in flours or meals, these changes also take place in the unground seeds.

Defatted soy bean meal stored for 1 year in bags at 24.4° decreased 18 per cent in the amount of nitrogen extractable by 10 per cent NaCl solution, 21 per cent in digestibility, and nearly 12 per cent in true protein value. Materially lower percentage changes occurred at −1.1°.

Denaturation of the proteins of wheat and of wheat flours occurred to a much larger extent and more rapidly than that of the soy bean proteins. After 1 month's storage in sealed jars at 24.4°, the nitrogen extractable from white flour by 3 per cent NaCl had decreased 43 per cent. At the end of 1 year, the percentage had dropped to approximately 50 per cent. Large decreases in the amount of nitrogen extractable with 70 per cent alcohol and Na salicylate also occurred, with similar changes occurring in whole wheat flour. Indications of extensive proteolysis in the wheat flours were shown by increased values in amino nitrogen and decreases in true protein values and in nitrogen precipitable by trichloroacetic acid.

Changes also occurred in the whole, unground wheat kernels. After storage in jars at 24.4° for 9 months, the percentages of nitrogen extractable by NaCl, alcohol, and sodium salicylate had decreased 26, 18, and 9 per cent, respectively. Considerable proteolysis had also occurred.

Sulfanilamide Metabolism in Cystinuria. By BEATRICE KASSELL, LEONARD PEARLSTEIN, ERWIN BRAND, AND GEORGE F. CAHILL. *From the Departments of Biological Chemistry and of Urology, College of Physicians and Surgeons, Columbia University, New York*

To determine the effect of sulfanilamide on protein and sulfur metabolism, and to investigate the fate of the sulfur of sulfanilamide, 8 gm. were fed to cystinuric patient A over a 3 day period (2, 2, and 4 gm.). The patient was maintained on a constant weighed diet. Free and acetyl sulfanilamide was determined by Marshall's* revised method, total sulfur by Rutenber and Andrews'† procedure, and other urinary constituents as previously described.

During and after the administration of the drug, there was no change in the blood picture. There was some toxic action, indicated by subjective complaints and by a stimulation of nitrogen metabolism, resulting in an increased excretion of total nitrogen. Creatinine and creatine excretion were constant throughout the experiment.

The excretion of cystine and of inorganic and ethereal sulfates remained at the control levels, indicating that sulfanilamide had no influence on sulfur metabolism. The extra neutral sulfur excreted exactly balanced the sulfanilamide fed: 39 per cent was free sulfanilamide, 52 per cent acetyl sulfanilamide, and 9 per cent (excreted during the feeding period) an unknown sulfur compound.

Within 48 hours of the last dose of sulfanilamide, 83 per cent had been eliminated, but the remainder was excreted very gradually over a period of 7 days. The proportion of free to acetyl sulfanilamide excreted was much greater during feeding than in the after period. This finding suggests storage of sulfanilamide in some combination from which it is liberated slowly and excreted as the acetyl compound.

---

* Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, **88**, 86 (1938).

† Rutenber, C. B., and Andrews, J. C., *J. Biol. Chem.*, **120**, 203 (1937).

A Quantitative Study of the Effect of Compounds with Cortin-Like Activity.

By Edward C. Kendall. *From the Division of Biochemistry, The Mayo Foundation, Rochester, Minnesota*

A quantitative investigation of the physiological activity of extracts and crystalline compounds separated from the adrenal cortex has been made on adrenalectomized dogs. Compound B (corticosterone) has been used as the reference compound. The minimal amount injected daily which is sufficient to maintain a normal condition of the animal with a blood urea between 45 and 55 mg. per cent is considered the requirement for each adrenalectomized dog. For satisfactory results a uniform diet low in potassium and sodium and high in vitamin B_1 content is necessary. The daily requirement of Compound B for a series of dogs varied between 1 and 3 mg. The requirement per kilo of body weight varied between 0.06 and 0.18 mg. Desoxycorticosterone was from 6 to 10 times more active and desoxycorticosterone acetate was from 15 to 20 times more active than Compound B. The most active amorphous fraction separated from the gland was about 40 times more active than Compound B.

The highly toxic nature of compounds with cortin-like activity can be demonstrated on rats only when there is a deficiency of insulin. In this condition the active extract produces a marked glycosuria and a negative balance in potassium and death may follow within 12 days the daily oral administration of the extract prepared from 750 gm. of adrenal glands.

Cardiac Output As Measured by Roentgenkymography.

By Ancel Keys and H. L. Friedell. *From the Departments of Physiology and Radiology, University of Minnesota Medical School, Minneapolis*

With the roentgenkymograph, equipped with a moving film and a stationary lead grid, the systolic and diastolic projections of the heart are traced on the x-ray film. The frontal projection made with a 2.5 second exposure from 6 feet, suitably corrected for distortion by lateral plates, gives an area measurement that is repeatable within a deviation of less than 1 per cent. From comparison of direct and x-ray measurements on cadavers of adults, the corrected frontal projection on the x-ray plate is related to the heart volume by the equation, volume (in cc.) = 0.63 × (area in sq. cm.)$^{1.45}$. Applicability of this kind of equation to the measurement of cardiac output was proved in roentgenkymographs made on dog heart-lung preparations. In twelve series of experiments on trained human subjects roentgenkymographs were made immediately before and after cardiac output measurement by the acetylene method. The results fit the equation, diastolic volume minus systolic volume = 0.99 × acetylene stroke output. The average discrepancy was ±3.5 per cent and the maximum error, with the acetylene result as standard, was − 6.8 per cent. The effects of coronary and pulmonary circulations are balanced by phase differences between contractions of the chambers and the particular components recorded in the frontal position. Values obtained for the cardiac output by roentgenkymography alone on several hundred resting normal subjects are very similar to those reported in the literature for the acetylene method with comparable subjects and conditions. In mitral insufficiency or aortic regurgitation, comparison of the two methods gives a quantitative measure of the mechanical inefficiency of the heart. The roentgenkymographic method may be applied to persons who are untrained or incapable of cooperation.

The Importance of Riboflavin and Ascorbic Acid in the Utilization of Vitamin A. By Marian S. Kimble and Edgar S. Gordon. *From the Department of Medicine, University of Wisconsin, Madison*

Individuals who show low normal or pathologically deficient biophotometer readings, and low blood vitamin A levels, can usually be restored to the normal range by the administration of vitamin A concentrates. The rather common occurrence of failure to improve biophotometer performance and blood vitamin levels by this means, however, has led to the belief that this instrument measures only one function of vitamin A, and that other unknown factors are involved in the utilization of the vitamin for the synthesis of visual purple. Both riboflavin and ascorbic acid have been suggested as these extra factors. Several otherwise normal individuals with low biophotometer readings and low blood levels were given pure riboflavin, 1 mg. daily, along with 200,000 units of vitamin A daily, after having gone through a control period of 2 months on the haliver oil alone. The combination immediately restored the biophotometer readings and blood to normal, when the vitamin A alone had failed to do so. In some other individuals in whom riboflavin failed to produce this result, ascorbic acid caused satisfactory improvement.

The Determination of Added Maltose in the Presence of Glucose in Blood, Tissue, and Urine. BY ALFRED E. KOEHLER, NORMAN MARSH, AND ELSIE HILL. *From the Santa Barbara Cottage Hospital and the Sansum Clinic, Santa Barbara*

The principle of the method is based on the relatively low concentration and instability of maltase in bakers' yeast (*Saccharomyces cerevisiæ*, Fleischmann) as compared with its glycolytic activity.

If thoroughly washed yeast is aged in the refrigerator for 4 or 5 days and again thoroughly washed, it has lost nearly completely its ability to ferment any appreciable amount of maltose at 37.5° in 10 minutes, while its ability to ferment glucose is still very good.

The usual procedure for yeast fermentation is followed with the specially prepared yeast, except that fermentation is carried on at 37.5° for exactly 10 minutes. For blood the Folin-Wu method of precipitation is used. The fermentable sugar is calculated as glucose and the non-fermentable as maltose is calculated against a maltose standard.

Maltose added to whole blood or urine can be recovered to the extent of about 98 per cent even from diabetic blood with high glucose content. In a large number and great variety of cases no evidence has been obtained to indicate the presence in blood or urine of a coenzyme that would increase maltase activity in the specially prepared yeast.

Different preparations of yeast vary in their maltase activity and after washing and aging it should be tested to see that it will not appreciably split maltose and that its glycolytic action is unimpaired.

This method is of course not specific for maltose but includes all non-fermentable substances. It has value, however, in maltose tolerance tests, in which only maltose is added, in demonstrating the absence of maltose and in studying the glucose-maltose-glycogen cycle.

Maltose Utilization in Diabetes Mellitus. BY ALFRED E. KOEHLER, EMANUEL WINDSOR, AND ELSIE HILL. *From the Department of Medicine of the Santa Barbara Cottage Hospital, the Santa Barbara General Hospital, and the Sansum Clinic, Santa Barbara*

These studies depend upon a modified fermentation method for the determination of maltose in the presence of glucose in biological material.

In macerated liver at 37.5° glycogen disappears rapidly, forming maltose; the latter then changes more slowly to glucose.

The comparative utilization of glucose and maltose was studied in diabetic and non-diabetic subjects by intravenous glucose and maltose tolerance tests, 100 gm. of each sugar being given with a constant rate pump over a period of 2 hours.

In eight cases of mild to moderately severe diabetes maltose was utilized better than glucose. In ten non-diabetic subjects having normal glucose tolerance curves, maltose was utilized as well as or not as well as glucose so far as blood rise and urinary excretion were concerned.

In eight cases of diabetes the average blood maltose level during and after injection was slightly lower than the average for the non-diabetic group. As compared with the control group the average excretion of urinary maltose in diabetes also indicated equally good or better utilization of maltose. One case of severe insulin-resistant diabetes utilized maltose poorly but utilized it better with insulin.

Insulin (40 to 50 units) administered before or during maltose injection has little effect on the blood maltose level or urinary excretion. In some cases insulin actually increases the blood maltose level and increases urinary excretion.

From these studies a theory is presented that the difficulty in certain cases of diabetes lies at least in part in the conversion of glucose to maltose and that insulin plays a rôle in this step.

The Grass Juice Factor. By G. O. Kohler, S. B. Randle, and J. R. Wagner. *From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

Further studies have been made on the "grass juice factor," a relatively labile vitamin necessary for the normal growth of rats and guinea pigs. The latter were used as assay animals for fractionation studies. The basal deficient ration was mineralized winter milk.

It was found that the factor was precipitated from grass juice by the addition of acetone, and then removed from this precipitate by washing with acid acetone. When grass juice was shaken with a chloroform-amyl alcohol mixture, the juice proteins were removed, leaving the factor in the deproteinated juice. When this deproteinated juice was treated with norit, the filtrate retained practically all of the activity.

The grass juice factor was extracted from dehydrated grass by acid acetone but not by water, ether, or petroleum ether. Continuous peroxide-free ether extractions of neutral or acidified concentrates failed to remove the growth factor.

The most potent concentrates were active at levels of 40 mg. per day. This represents a 50-fold concentration.

Studies were undertaken to produce a dry ration deficient in the grass juice factor. The ration finally used had the following composition: dextrin 59, casein 18, salts 4, yeast 6, cod liver oil 1, lard 12. Each animal received 2 cc. of orange juice daily. Guinea pigs on this regimen died in 3 to 10 weeks. When 20 per cent of dehydrated grass was included, they grew normally.

Stomach ulcers were observed in animals on this ration, but apparently were not related to grass juice factor deficiency.

Chemical Studies in Mental Disease. The State of the Serum Proteins. By Albert A. Kondritzer. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York, and the Neuro-Psychiatric Institute of the Hartford Retreat, Hartford*

The use of convulsants in psychotic conditions has led to a search for the physiological accompaniment of the psychical results obtained. Harris and others have reported alterations in some of the inorganic blood constituents during insulin treatment. Preliminary to an extensive investigation on the serum proteins of psychotic individuals, it was thought advisable to investigate the effect of insulin and metrazole administration on the state of the serum proteins.

It has been suggested that there is an albumin-globulin disequilibrium in the blood of schizophrenic individuals. These findings were based upon the conventional methods of precipitation of the blood proteins with an arbitrary concentration of neutral salt. Perlzweig, Kondritzer, and Bruch showed that this method often fails to yield significant results. Cohn's procedure of fractional precipitation with gradually increasing amounts of salt is more valuable. The modification of Butler and Montgomery's method with potassium phosphate was used in this study. Serum proteins from patients on insulin and metrazole therapy were studied. Blood was obtained immediately before injection of the metrazole and just after the conclusion of the convulsion. In the case of the insulin patients, blood was removed during the comatose state. Sera from normal individuals and psychotic patients on "insulin control" were also investigated.

The data, at present, fail to indicate any apparent deviation from the normal in the solubility precipitation pattern of the serum proteins of schizophrenic and psychoneurotic patients before, during, or after insulin and metrazole treatment.

Creatine-Creatinine Metabolism and the Hormones. II. Progestin and Antuitrin-T.

By A. Link Koven and Howard H. Beard. *From the Department of Biochemistry, School of Medicine, Louisiana State University, New Orleans*

In a previous study (Pizzolato and Beard*) it was shown that castration of rats of both sexes results in creatinuria. The injection of theelin, follutein, testosterone propionate, and anterior pituitary hormones (G, T, S) caused increases in muscle creatine in normal animals, which were followed by increases in creatine excretion accompanied by decreases in creatinine excretion. The creatinuria was more intense in the castrated than in the normal animals.

---

* Pizzolato, P., and Beard, H. H., *Endocrinology*, in press.

These findings have been further confirmed in the present studies. Progestin (commercial and crystalline) and antuitrin-T were injected into normal and into castrated rats. Creatinuria was more intense in the castrated animals after injection of the crystalline progestin than after the injection of the commercial variety. The creatinuria after injection of antuitrin-T was likewise more intense in the castrated than in the normal animal. Neither castration nor the injection of commercial progestin and antuitrin-T affected the content of muscle creatine in either the normal or the castrated animal.

Harrop† has shown that injection of the sex hormones causes an intense retention of Na^+ in the dog. Our results indicate that this retention causes an increase in water retention, which in turn favors the hydrolysis of creatinine to creatine. The retention of creatinine observed in this study after the injection of hormones and the fact that the creatinine → creatine transformation does not occur in castrated animals furnish further support for this view.

Apparent Dissociation Constants of Thirty Substituted Barbituric Acids As a Function of Ionic Strength and Temperature.

By M. E. KRAHL. *From the Lilly Research Laboratories, Indianapolis*

In the course of a study of the mechanism of action of pharmacologically active acids and bases, the effects of variation in NaCl concentration on the pK′ values of thirty substituted barbituric acids have been determined at 25° from glass electrode pH measurements. With concentrations of NaCl up to 2 M, the relation between the pK′ values of these acids and the ionic strength can be approximately expressed by a form of the Debye-Hückel equation. By means of this equation pK values at 25° have been calculated for 30 di- and trisubstituted barbituric acids; these vary from 7.21 for 5-allyl-5-benzylbarbituric acid up to 8.34 for 5-cyclohexenyl-1,5-dimethylbarbituric acid.

The effects of variation in temperature on the pK′ and pK values of 5-isoamyl-5-ethylbarbituric acid have been determined at temperatures between 15° and 40° and the values of the standard free energy change, ΔF°, at each temperature have been calculated. For this acid in this temperature range the relation between ΔF° and temperature is expressed by the usual thermodynamic equations in which ΔS°, the standard entropy change, has a value of -3.1 calories per degree.

---

† Harrop, G. A., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, 5, 375 (1937).

By means of these experimental data and the equation

$$\mathrm{pK}'_T = \frac{\Delta F^\circ_{T_0} + 3.1\,(T - T_0)}{2.3\,RT} - \log \gamma_{A^-}$$

in which the symbols have their usual significance for the absolute temperatures T and T_0, approximate pK′ values of the thirty barbituric acids can be calculated for any ionic strength and any temperature in the physiological range.

The Determination of Quinine in Blood.

By Granvil C. Kyker and James C. Andrews. *From the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill*

A study is being made of the quinine content of blood after quinine therapy in malaria. Examination of the analytical methods available has indicated that precipitation and nephelometric determination of quinine silicotungstate, as described by Vedder and Masen, is preferable. This method has, therefore, received further study.

The acidity of the precipitating medium is the chief factor. Vedder and Masen recommend the use of 0.5 M HCl. However, a series of determinations on standard aqueous solutions of quinine in acidities ranging from 0 to 0.5 M HCl revealed that the completeness of precipitation was optimal at 0.03 M HCl. When acidity is plotted against apparent quinine concentration, the resulting curve rises sharply with acidity from 0 to 0.03 M HCl, and thence drops rapidly to 0.1 M. From 0.1 to 0.5 M the apparent recovery of quinine is approximately equal to that obtained in the absence of acid, and the sharp rise and fall exist entirely between 0 and 0.1 M.

The increase in sensitivity at the optimal acidity is approximately 60 per cent over that obtained with 0.5 M HCl. As modified, as little as 1 mg. of quinine per liter in aqueous solutions can be determined by the method. We could not obtain this degree of sensitivity with the unmodified method.

Smoother and more quantitative performance is obtained in removing the quinine from the sample by modifying the apparatus for continuous ether extraction.

The application of this modified procedure to the determination of quinine in blood is now in progress.

The Basal Metabolic Rate of the Eskimo. By Victor E. Levine.

From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha

Our previous report indicated that the basal metabolic rate of the Eskimo may well conform to standards in the temperate zone. Heinbecker and Rabinowitch found the rate to range from +26 to +33.

During our last sojourn in the Arctic from August, 1937, to August, 1938, made possible by a generous grant from the United States Public Health Service, we determined under more favorable and more ideal conditions the basal metabolic rate of eleven male and twelve Eskimo females at Point Barrow; the subjects were 11 to 46 years of age; six were one-eighth to one-fourth White. We secured sufficient data to judge emotional and physical normality. These data comprised complete medical examination, including pulse, temperature, blood pressure, a search for focal or systemic infection, urine analysis, erythrocyte count and hemoglobin determination to rule out anemia or polycythemia, leucocyte and differential count, tuberculin test, and x-ray of the chest. Emotional states, such as fear, anxiety, or apprehension, were minimized by personal acquaintanceship of many months duration and by the performance of various biologic tests and manipulations (intradermal tests, venipuncture, etc.).

Eighteen Eskimos gave normal rates, while one male and four females gave high rates. The results obtained under more ideal conditions confirm the previous finding that the basal metabolic rate of the Eskimo may be within the normal limits accepted in the temperate zone, and bears no relationship to race or climate.

Urinary Excretion of Vitamin C. By Herbert E. Longenecker, R. R. Musulin, and C. G. King. *From the Department of Chemistry, University of Pittsburgh, Pittsburgh*

The urinary excretion of vitamin C by rats varies greatly with different vitamin C-free rations. The primary cause of variation was located in the unsaponifiable matter of the diets. Chemical examination of these lipids led to the study of cyclic compounds of the terpene and sesquiterpene series.

Many compounds, *e.g.* carvone, isophorone, menthol, isoborneol, nerolidol, thujone, camphor, etc., when fed in doses of 10 to 100 mg., raise the level of vitamin C excretion from approximately 0.3 mg. per day on a milk diet to values varying from 2.0 to 17.0 mg. Feeding pure ascorbic acid in doses as high as 100 mg. produced excretions of only 5 to 7 mg. The presence of vitamin C in the urines was confirmed by guinea pig assays, the test levels being based upon titration values.

Cyclic and branched chain aldehydes, ketones, alcohols, and related substances have been studied previously in relation to glucuronic acid detoxication in dogs, rabbits, and man. Apparently no records of detailed feeding experiments and urine examinations with rats have been published. We have studied the possibility of conjugation with the vitamin and have reexamined the effect of feeding similar compounds to the rabbit and dog. No increase of vitamin excretion was observed in rabbits after menthol feeding but a definite rise was obtained in the dog.

The possibility that such compounds may serve as vitamin precursors in the rat and the possible function of vitamin C as a detoxicating agent will be discussed.

The Determination of Globulin and Albumin in Blood Serum by the Photoelectric Colorimeter. By Joseph M. Looney. *From the Memorial Foundation for Neuro-Endocrine Research, Worcester State Hospital, Worcester*

The method of Looney and Walsh* for the determination of spinal fluid protein has been adapted to the estimation of globulin and albumin in 1 cc. of blood serum. For the globulin determination 1 cc. of blood serum is diluted to 10 cc. with 1 per cent salt solution and 1 cc. of this solution is mixed with 2 cc. of 2 per cent gum ghatti and 3 cc. of saturated ammonium sulfate. The resulting turbid solution is read in the photoelectric colorimeter and the amount of globulin estimated by comparison with a standard curve obtained from different dilutions of a solution of serum globulin of known concentration.

---

* Looney, J. M., and Walsh, A. I., *J. Biol. Chem.*, **127**, 117 (1939).

A solution containing 1 per cent of serum in 1 per cent salt solution is prepared by diluting the first serum mixture again in the proportion of 1:10. The total protein is determined by mixing 2 cc. of this solution with 0.5 cc. of 2 per cent gum ghatti and 2.5 cc. of 5 per cent sulfosalicylic acid. The turbid solution is read in the photoelectric colorimeter and the concentration of protein obtained by comparison with a standard curve prepared by treating dilutions of blood serum of known concentration in a similar manner. The albumin is estimated by the difference between the two values.

It is necessary to wait until the mixtures become completely free from fine bubbles before reading. The turbid solutions are very stable and show no evidence of settling out up to 96 hours.

The Diffusion Constants and Approximate Molecular Weights of Tobacco Mosaic Virus Nucleic Acid and Yeast Nucleic Acid.

By Hubert S. Loring. *From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*

The diffusion constants of tobacco mosaic virus nucleic acid and yeast nucleic acid were determined in 0.4 M borate buffer at pH 7.7 by the method of Northrop and Anson. Values for D of 0.10 sq. cm. per day and 0.13 sq. cm. per day, respectively, were obtained. The calculation of molecular weights from these results by means of Einstein's equation, $D = RT/N6\pi r\eta$ gave values of 37,000 for the virus nucleic acid and 17,000 for yeast nucleic acid. In the case of yeast nucleic acid, examination of the material remaining in the diffusion cell after about 20 per cent of the nucleic acid had diffused showed the presence of a more slowly diffusing component with D of 0.11 sq. cm. per day. If the tetranucleotide structure generally accepted for pentose nucleic acid be assumed to represent only the basic structure, these results indicate that tobacco mosaic virus nucleic acid is made up of about twenty-nine and yeast nucleic acid of from fourteen to twenty-two tetranucleotide units. Whereas it has been generally assumed that pentose nucleic acids from different plant sources are identical, these results indicate that in these two cases they are different.

Factors Affecting the Distribution of Salts and Water in Muscle. By Oliver H. Lowry. *From the Department of Biological Chemistry, Harvard Medical School, Boston*

Rat muscle has been equilibrated anaerobically with balanced salt solutions at 0°, 6°, and 36°. The rate of exchange between the tissue and the solution of H_2O, Na, K, and Cl has been studied. The results have been compared with those from heated controls equilibrated at the same temperature. The rate at which equilibrium was reached was much slower for the normal muscle than for the controls, and slower at the lower than at the higher temperatures.

The significance of those results for the interpretation of analytical data in terms of the extra- and intracellular phases of muscle will be discussed.

The Effect of Partial Hepatectomy on the Liver Lipids. By Stephan Ludewig. *From the Biochemical Laboratory, University of Virginia, University*

Following partial hepatectomy (70 per cent removal), the lipid chemistry of the rat liver was studied at frequent intervals. At the end of 24 hours, the total fatty acid, the phospholipid fatty acid, and cholesterol concentrations increased markedly. On the 2nd day, the concentration of the total fatty acids and phospholipid fatty acids decreased slightly but the cholesterol concentration remained high. Mitotic activity is most marked on the 3rd day and was accompanied only by a decrease in the total fatty acids. After this time, all constituents studied decreased somewhat but did not reach the control levels on the 7th day. The iodine number of the total fatty acids was unchanged but there was a gradual increase in this value for the phospholipid fatty acids.

Oxidations Produced by the Kidney in Experimental Nephritis. By Carl M. Lyman and E. S. Guzman Barron. ***From the***

Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago

The kidney draws from oxidative processes the energy necessary for the performance of its manifold activities. When its functions are impaired (as in nephritis), there may be produced alterations of the oxidative processes which in turn may further impair the ability of the kidney to perform work. The cells of the tubules, because they take part in the important functions of secretion and absorption, might suffer greater damage in their metabolism than those of the other regions of the kidney. The rate of oxygen consumption of kidney slices of control rats was compared with that of rats poisoned with diethylene glycol (which produces acute nephritis with profound damage of the tubules). No difference was observed until oxidizable substrates were added, whereupon the nephritic kidney showed a marked inhibition in the rate of oxidation of these substrates. The oxidations of pyruvic acid and choline were inhibited to the highest degree (about 91 and 95 per cent respectively). Lactate oxidation was inhibited by about 85 per cent; alanine and aspartic acid oxidation by 66 per cent; succinic acid oxidation by 43 per cent. The formation of NH_3 was also diminished by 32 per cent.

The inhibiting effect produced by diethylene glycol seems to be due in most cases to its action on the activating proteins (dehydrogenases) of these oxidizing enzymes. This opinion is supported by studies on the rate of anaerobic oxidation with ferricyanide in bicarbonate buffer as the oxidizing agent. Under such conditions there will be reduction of ferricyanide only when the substrate has been activated by the activating protein of the enzymes. In the nephritic kidney the anaerobic oxidation of lactic acid was almost nil; the oxidation of choline was inhibited by 25 per cent; the oxidation of succinic acid by 62 per cent.

Normal Creatine, Phosphorus, and Potassium Content of Human Cardiac and Voluntary Muscle.*

By GEORGE MANGUN AND VICTOR C. MYERS. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

Most of the values appearing in the literature for creatine, phosphorus, and potassium in human cardiac and voluntary muscle have been based upon miscellaneous autopsy cases. In the course of studies carried out in this laboratory it seemed desirable to obtain figures from cases that could be classified as strictly normal. A series of cases of sudden death was therefore obtained within 24 hours post mortem. The creatine, phosphorus, and potassium of the pectoralis major ranged in eleven cases from 426 to 462 (average 443), 195 to 216 (average 201), and 311 to 346 (average 328) mg. per 100 gm. of fresh tissue respectively. These values, particularly for creatine, are somewhat higher than had been anticipated upon the basis of miscellaneous autopsy cases. Our previous estimate[†] of 425 mg. as the probable creatine saturation level of voluntary muscle therefore appears to be slightly low. Miscellaneous autopsy cases in three separate studies from this laboratory have yielded averages very close to 400 mg. In thirteen cases, the left and right ventricles of the heart yielded results within the range anticipated from previous studies. The creatine, phosphorus, and potassium ranged from 186 to 218 (average 203), 180 to 204 (average 194), 251 to 305 (average 285) mg. per 100 gm. respectively for the left ventricle, and 154 to 185 (average 165), 140 to 187 (average 160), and 189 to 242 (average 219) mg. per 100 gm. respectively for the right ventricle.

---

* Aided by a grant from the Josiah Macy, Jr., Foundation.

Investigations on Surface Tension and Enzyme Activity. By T. Urban Marron and Ferrin B. Moreland. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

The effect of several surface-active agents (methyl, ethyl, propyl, butyl, and amyl alcohols; digitonin, saponin, and alphasol MA) on the activity of phosphatase, urease, and tyrosinase was studied. There was no significant relation between surface tension and enzyme activity.

Blood Iron Studies in the Fetal Guinea Pig. By Morton F. Mason. *From the Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville*

The partition of blood iron in the fetal guinea pig at various stages of intrauterine life has been studied in respect to the following fractions: total iron, "easily split" iron including Fractions E and E′ (Barkan), and total serum iron. The findings have been compared with the simultaneously determined values in the maternal blood. Observations have also been made on the changes in iron stores of the fetal and maternal guinea pig during pregnancy.

[†] Linegar, C. R., Frost, T. T., and Myers, V. C., *Arch. Int. Med.*, **61**, 430 (1938).

Blood Hormone Iodine and Iodide Reserve in Relation to Basal Metabolic Rate.

By J. F. McCLENDON AND CARL O. RICE. *From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis*

Since we observed that 2 grains of desiccated thyroid per day did not raise the metabolic rate (statistically) of thyrotoxic patients, some chemical means of determining the thyroid hormone in the blood seemed desirable. We define the iodine that may be extracted with methanol followed by acetone as the iodide reserve and the remainder as hormone iodine. Statistically the hormone iodine in 5 cc. of blood = 0.01 (× the basal metabolic rate in Calories per sq. m. per hour − 20). The iodide reserve of persons not taking iodide medication averages 0.01 × (60 minus the Calories per sq. m. per hour); in other words, statistically the iodide reserve vanishes when the basal metabolic rate reaches about 60 Calories per sq. m. per hour in this region of relatively low iodine intake. We believe this is due to two causes; first the transformation of some of the iodide reserve into hormone iodine, and second the increased sweating and urea formation resulting in increased elimination of iodide in sweat and urine. It seems probable that the disappearance of the iodide reserve is dangerous, although we have not actually determined it in patients dying in thyroid crisis.

The fact that the "hormone iodine" varies directly with the basal metabolic rate indicates that it is an actual measure of thyroid hormone. Such a demonstration has not been made by previous workers.

Effect of Castration on Blood Androgens.

By D. ROY McCULLAGH AND ROBERT DAOUST. *From the Department of Biochemical Research, Cleveland Clinic, Cleveland*

By the method of McCullagh and Osborn* the androgens in the blood of castrated and normal rabbits have been studied. Approximately 40 cc. of blood were drawn from each animal. The androgenic extract of each sample of blood was assayed by use of five capons. The extracts from thirteen normal adult male animals caused an average comb growth of 3.8 mm. (increase in length plus height). Four similar animals were castrated for periods varying from 3 to 25 days. Extracts of their blood caused growth of only 1.7 mm. The blood from five animals castrated from 1 to 5 months caused 4.0 mm. of comb growth, and that from nine animals castrated more than 2 years an average growth of 3.7 mm. The origin of the androgenic material which appears in the blood of castrated rabbits has not been ascertained. The atrophic condition of the secondary sex glands of the castrated animals indicated that the androgenic material in the blood of castrates is in a physiologically inactive form. Activation probably occurs as the result of boiling with acid prior to extraction. It is possible that activation of androgens produced elsewhere is a normal function of the testes.

---

* McCullagh, D. R., and Osborn, W. O., *J. Biol. Chem.*, **126**, 299 (1938).

The Effect of Several B Vitamins and of Choline upon Liver and Body Fat.

By E. W. McHenry and Gertrude Gavin. *From the School of Hygiene, University of Toronto, Toronto, Canada*

In rats and pigeons fed upon a high carbohydrate, fat-free diet thiamine causes an increased amount of fat, principally in the liver. The new fat is presumably formed from carbohydrate. The amounts of fat in the liver and in the body may be altered by various dietary supplements. Riboflavin, as the only supplement, has no definite effect upon liver or body fat; in conjunction with thiamine it causes a further increase in liver fat. Adequate amounts of choline, which is as active parenterally as by mouth, reduce the liver fat but fail to increase body fat. The latter can be increased by supplying a source of vitamin B_6 (rice polish concentrate). These results have been verified in paired feeding experiments.

Purification of the Choline Esterase of Serum and the Crystallization of Serum Albumins.

By Thomas L. McMeekin. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The presence of choline esterase in serum has been adequately demonstrated.* In connection with our studies on the characterization of the proteins of serum it seemed desirable to concentrate and purify choline esterase.

Both normal horse serum and antipneumococcus horse serum have been fractionated with equal facility. A fraction of the serum proteins was first removed at one-third saturation of ammonium sulfate, and a second fraction at half saturation of ammonium sulfate. These fractions contained very small quantities of esterase and could be practically freed therefrom by reprecipitation.

Most of the serum albumin could be crystallized and removed by carefully acidifying the filtrate from the globulin fractions in the presence of half saturated ammonium sulfate. It was found that the albumins could be separated more effectively by allowing the ammonium sulfate to diffuse through a rotating cellophane membrane. By this procedure choline esterase has been purified so that its activity is about 100 times greater per unit of weight than the original total serum protein. A further purification was effected by means of lead acetate. The esterase was not precipitated in dilute solution by lead acetate at pH 7.0, but was precipitated at pH 7.4. This treatment increased the esterase activity to 150 times that of serum. The purest preparation of esterase was protein in character, and did not contain a carbohydrate group.

At least three crystalline albumins have been separated from serum, each with characteristic crystalline form. These observations indicate further† the complexity of even the repeatedly recrystallized serum albumin fraction.

Enzymic Oxidation of Sulfur-Containing Amino Acids. By GRACE MEDES. *From the Lankenau Hospital Research Institute, Philadelphia*

Cysteine, cystine, cystine disulfoxide, and cysteine sulfinic acid are oxidized with formation of inorganic sulfate in the presence of tissue slices. The results with cysteine and cystine agree in general with those obtained by Pirie.* Both liver and kidney slices are highly active; blood, spleen, and lung tissue are inactive, while cardiac and heart muscle possess a low degree of activity. Sulfate formation from cystine by liver and kidney tissue was considerably less than from cysteine. The yield of sulfate from cystine disulfoxide was of the same order as from cystine. Oxidation of cysteine sulfinic acid occurred most readily, the yield in all cases being higher than with any of the other compounds.

---

* Stedman, E., and Stedman, E., *Biochem. J.*, **29**, 2563 (1935).

† Sörensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, **12**, 12 (1917). Ferry, J. D., and Oncley, J. L., *J. Am. Chem. Soc.*, **60**, 1123 (1938).

Extraction or partial purification of the enzymes concerned in these oxidations has demonstrated the presence of at least four enzymes: (*a*) *cytochrome oxidase*, which oxidizes cysteine quantitatively to cystine. It has no effect on cystine, methionine, cysteine sulfinic acid, or cysteic acid; (*b*) a second oxidase (*cysteine oxidase*) which oxidizes cysteine to cystine. This reaction does not take place quantitatively, only 50 to 90 per cent of the cysteine being recovered as cystine, depending upon the conditions of the experiment. Cystine slowly disappears under the action of this enzyme. Its oxidation products have not yet been identified. No inorganic sulfate is formed and apparently no sulfinate; (*c*) an oxidase (*sulfinic acid oxidase*); (*d*) an oxidase which converts cysteine to inorganic sulfate.

Methods of extraction and identification will be presented.

Urinary Excretion of Thiamine. By DANIEL MELNICK AND HENRY FIELD, JR. *From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor*

Our recently published method for the chemical determination of thiamine, based on the reaction between the vitamin and diazotized *p*-aminoacetophenone, has been applied to analyses of urine. Na_2SO_3 is added to the urine sample, adjusted to pH 4.5, and the solution is concentrated *in vacuo* until salts begin precipitating. Benzyl alcohol is then added and the distillation continued until most of the water is removed. The alcoholic solution is shaken with acidulated water and ether, the vitamin passing into the aqueous phase. The latter is subjected to zeolite adsorption with subsequent elution. The eluate is then suitable for the chemical test.

---

* Pirie, N. W., *Biochem. J.*, **28**, 305 (1934).

Tests conducted with the entire 12 hour urine samples give consistent recoveries of from 85 to 95 per cent of added thiamine. With urine samples of 6 hours or less, theoretical recoveries of added vitamin are obtained. The method is highly specific for thiamine in urine. No other substances, including a large number of drugs, have been found to yield a color which is extracted by the xylene. In normal urines no appreciable amounts of phosphorylated thiamine were found. The stability of the vitamin in urine at different physiological pH values, its excretion by normal and avitaminotic individuals before and during the parenteral administration of extra vitamin, the diurnal variation and its significance, and factors influencing the "saturation" test were studied.

Blood Cholesterol in Rabbits during Experimental Herpes Virus Infection Treated with Vitamin B_1 and with Sulfanilamide. By Samuel Member and Maurice Bruger. *From the Department of Medicine, New York Post-Graduate Medical School, Columbia University, New York*

Daily determinations of whole blood cholesterol were carried out on thirty-one rabbits with experimental herpes virus infection. Of these, ten received 20 mg. of vitamin B_1 intramuscularly daily, fourteen sulfanilamide or its derivatives in daily doses of 1 or 2 gm. orally or intramuscularly, and seven rabbits served as controls.

Herpes virus infection in rabbits is accompanied by no consistent alteration in the blood cholesterol in spite of a uniform rise in body temperature (rectal) to 41.11° in all animals. The administration of vitamin B_1 and of sulfanilamide or its derivatives to rabbits with herpes virus infection likewise did not consistently influence the blood cholesterol. For the most part, these agents accentuated the variations observed but in no uniform direction.

Endogenous Citric Acid Production. By Curtis E. Meyer. *From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*

Recent reports in the literature offer evidence for the endogenous origin of urinary citric acid. Additional data in support of this thesis have been obtained. The citric acid balances of rats maintained on various types of diets extremely low in this compound have been determined. Animals which received a diet high in protein remained practically in balance over a period of 18 days, while those maintained on a high carbohydrate or high fat ration excreted as much as 30 mg. a day more than they ingested. This production of citric acid is not dependent upon the pH of the urine.

The Isolation of a Mucopolysaccharide from Synovial Fluid.

By Karl Meyer, Elizabeth M. Smyth, and Martin H. Dawson. *From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, and the Institute of Ophthalmology and the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York*

In continuation of former work on mucopolysaccharides and glycoproteins, the so called mucin of synovial fluid was investigated. By a method similar to that used in the isolation of chondroitinsulfuric acid,* a polysaccharide acid of high molecular weight was isolated from cattle synovial "mucin."

This polysaccharide acid was proved to be identical with the acids formerly isolated from vitreous humor, umbilical cord,† and Group A hemolytic streptococcus in the mucoid phase.‡ The composition and rotation of the acids were very similar, and the acids were hydrolyzed at the same rate by a specific enzyme isolated from pneumococcus and more recently from streptococcus. Experiments to obtain antisera against the polysaccharide acids were unsuccessful. No indication was found for the presence of chondroitinsulfuric acid. The acid occurs in the synovial fluid either free or as a protein salt.

The protein compound formed on acidification of synovial fluid apparently is a salt of a globulin with the acid. By salting-out with ammonium sulfate in weakly ammoniacal solution, the components are separated and obtained free of each other.

A Specific, Enzymatic Method for the Analysis of Creatine and Creatinine in Tissues, and Its Application to Studies of the

---

* Meyer, K., and Smyth, E. M., *J. Biol. Chem.*, **119**, 507 (1937).

† Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **114**, 689 (1936).

‡ Kendall, F. E., Heidelberger, M., and Dawson, M. H., *J. Biol. Chem.*, **118**, 61 (1937).

Distribution and Interrelationship of Creatine and Creatinine in Mammalian Tissues.*

By Benjamin F. Miller, Zelma Baker, and M. J. Carl Allinson. *From the Department of Medicine, the University of Chicago, Chicago*

A specific, enzymatic method for the estimation of creatine and creatinine in tissues has been developed. Creatine is converted to creatinine by dehydration with hot acid, and the creatinine produced is determined colorimetrically with alkaline picrate before and after incubation with the bacterial enzymes previously described by Miller and Dubos for the specific decomposition of creatinine. Endogenous creatinine is extracted from the tissues with hot water and similarly determined. The colorimetric estimations are made in the Evelyn photoelectric colorimeter or the Pulfrich stufenphotometer which permit the precise estimation of as little as 0.2 microgram per cc. of final extract.

These methods have been applied to a study of the creatine and creatinine content of various tissues of the rat, dog, and monkey and have uncovered a marked discrepancy between the true and "apparent" creatine and creatinine contents of many tissues, particularly liver. In muscle, testis, and brain the true creatine corresponds fairly closely with the "apparent" creatine, whereas in most other tissues the true creatine varies from about 15 to 70 per cent of the apparent values. In all tissues studied there exists a rough proportionality between the concentration of true creatine and creatinine.

Comparison of Several Calcium Salts As to Their Effect upon Lactose Utilization.

By Helen S. Mitchell, Gladys M. Cook, and Katherine L. O'Brien. *From the Nutrition Laboratory, Massachusetts State College, Amherst*

The relative merits of the various calcium salts used in present day calcium therapy have recently attracted some attention. Repeated observations in this laboratory indicate that the gluconate differs from other calcium salts in its specific effect upon the digestion and absorption of lactose.

Rats were fed an adequate ration containing 60 per cent lactose, plus 1.0 or 0.5 per cent of calcium added in the form of six different calcium salts, respectively, tricalcium *phosphate, carbonate, citrate, lactate, levulinate,* and *gluconate.* Rats on any of the first five calcium salts showed as good growth, less diarrhea in general, about the same degree of galactemia, and a similar incidence of cataract as on the plain 60 per cent lactose ration. With *calcium gluconate* fed at the 1.0 per cent calcium level, few rats survived; these grew but little, had severe diarrhea, low blood sugar, and no lenticular changes whatever. With 0.5 per cent calcium (5.6 per cent calcium gluconate), the survival was better, growth poor, diarrhea moderate, lenticular changes few if any. These criteria all indicate that calcium gluconate prevents most of the lactose from leaving the intestinal tract. Since calcium gluconate shows no inhibitory effect upon the absorption of the single sugars *glucose* or *galactose,* the problem must concern the breakdown of the disaccharide. *Sodium gluconate,* fed at corresponding levels, exerted a similar deleterious effect, with only one out of eight rats surviving the experiment.

---

* Aided by a grant from the John and Mary R. Markle Foundation.

It would seem that the gluconate radical in some way inhibits lactase activity. For this reason, the therapeutic use of calcium gluconate and lactose together is questioned.

Hypervitaminoses D_2 and D_3 in Rats As Affected by Calcium and Phosphorus of the Diet. By AGNES FAY MORGAN, JEANETTE B. HENDRICKS, AND NOBUKO SHIMOTORI. *From the Laboratory of Home Economics, University of California, Berkeley*

Moderately excessive doses, 4000 to 10,000 U. S. P. units per day, of irradiated ergosterol (vitamin D_2) and fish liver oil concentrates (vitamin D_3) given to young rats on a basal diet containing adequate amounts of calcium and phosphorus were found previously to produce more markedly toxic results in the groups given vitamin D_2 and relatively low amounts of vitamin A. A similar comparison has now been made on rats fed the same basal diet and on parallel groups fed diets of low P-adequate Ca and adequate P-low Ca content for 27 to 32 days. Parathyroid extract, 0.4 unit per gm. of body weight daily, was injected into an additional subgroup in each case also. The vitamin A intake of all groups was the same.

All groups given the normal diet, that is adequate Ca-adequate

P, except those receiving excess vitamin D_2, grew normally and had normal femur ash and soft tissue Ca content. The group which received excess vitamin D_2 had severe symptoms of hypervitaminosis. The rats on the low P-adequate Ca diet grew very little and exhibited severe symptoms of hypervitaminosis in the group on excess vitamin D_2 and to a lesser extent in those on excess vitamin D_3. Those on the low Ca-adequate P diet on the other hand grew nearly normally and had no symptoms of hypervitaminosis except that the femur ash content was low. The parathyroid extract had no effect on bone ash in any of the groups and none on tissue Ca except for the kidney. Thus the Ca and P content and ratio in the diet were found to be of primary importance in determining the response to hypervitaminosis D and to parathyroid extract in regard to growth, food utilization, serum Ca, femur ash, and soft tissue Ca, particularly of the kidney.

The Effect of Phosphate Administration on the Capacity of the Dog for Work. By Minerva Morse. *From the Department of Pediatrics, the University of Chicago, Chicago*

A study of the effects of phosphate administration on the capacity of the dog to run on the treadmill has included doses of 4.0 gm. of $NaH_2PO_4 \cdot H_2O$ immediately, ½, 1, or 2 hours before exercise or daily for at least a week, 4.0 gm. ½ and 1 hour before, 10.0 gm. 15 hours before, 7.8 gm. of $Na_2HPO_4 \cdot 12H_2O$ immediately or 1 hour before, and a mixture of the acid and dibasic phosphate of pH 7.4 1 hour before. Administration of phosphate has shown little influence on capacity for work as measured by the length of the run.

During the first 15 minutes after ingestion, exercise appears to promote absorption of phosphate; as exercise continues, serum phosphate falls rapidly. If phosphate is given immediately or 1 hour before exercise fatigue may occur when serum phosphate is normal. The low serum phosphate which is usually found after exercise would not seem to be a causal factor in fatigue. Daily doses of acid phosphate neither raise the initial serum phosphate level nor prevent the decrease in serum phosphate which occurs in the first half hour of recovery.

A determination of changes in other blood constituents during exercise, pH, bicarbonate, lactate, chloride, total base, potassium, protein, and sugar has shown no consistent influence of phosphate administration.

Electrolyte and Water Exchanges between Muscle and Blood in Adrenal Insufficiency. By Edward Muntwyler, Frederick R. Mautz, George Mangun, and Robert C. Mellors. *From the Departments of Biochemistry and Surgery, School of Medicine, Western Reserve University, Cleveland*

A study of the electrolyte and water content of skeletal muscle (lumbar portion, sacrospinalis) obtained from a series of normal dogs on a constant diet yielded the following average results (expressed in terms of 1000 gm. of fat-free muscle): chloride 19.3, sodium 28.0, and potassium 82.6 milliequivalents, and water 761.2 gm. The average serum chloride was 116.5 milliequivalents per 1000 gm. of water. By assuming that the chloride ion is limited to the extracellular phase, the average extracellular phase (F) is calculated to be 159.0 gm. per 1000 gm. of muscle. The average collagen N (determined as gelatin N) was found to be 5.1 gm. per 1000 gm. of fat-free muscle. If the latter value is taken and certain assumptions employed, it is estimated that the average extracellular phase becomes increased to approximately 190.0 gm. per 1000 gm. of muscle.

Muscles from dogs in adrenal insufficiency showed a lowered chloride and sodium content. The decrease of sodium was relatively greater than that of chloride. The muscle potassium was increased slightly and there was an appreciable increase of the water content. By assuming a constant solid content of the intracellular phase of the control and experimental muscles, it is calculated that, relative to the control, the bulk of the muscle increases in insufficiency. This is the result of an increase of the amount of the intracellular phase in muscle and a relatively smaller decrease of the amount of extracellular phase.

Constancy of the Chemical Composition of Serum Proteins Regenerated under Various Dietary Régimes. By William A. Murrill and L. H. Newburgh. *From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor*

Total protein was prepared for analysis from the serum of normal dogs and from the same animals while subjected to quantitative plasmapheresis.* During this régime the dogs subsisted either on an artificial, "synthetic" protein-free diet or on a ration whose protein was furnished from a single dietary source (beef serum protein, casein, or lactalbumin). The proteins analyzed represented pooled aliquots of the serum proteins of blood removed in the bleedings of the equilibrium week, following an adjustment period of 1 week. Calculations have indicated that approximately 80 per cent of the serum proteins was regenerated in each case while the animal subsisted on the specified diet.

The normal serum proteins of this series contained 1.8 per cent histidine, 5.7 per cent arginine, and 9.0 per cent lysine, or, expressed in molecular ratios, 3.5:10:19. These results are in agreement with those reported by Block. The values obtained in the analyses of all the samples of protein collected in the plasmapheresis experiments were essentially the same.

The present findings support the contention of Block, as expressed in his "anlage" theory, that the total serum proteins are constant in composition, in so far as the basic amino acids are concerned. Despite the marked regeneration of plasma proteins occasioned by the plasmapheresis, no evidence of lability of serum proteins was obtained.

The Effect of Phosphorus on the Biological Estimation of Vitamin D Activity. By Brian O'Brien and Kenneth Morgareidge.

From the Institute of Optics and the Department of Biochemistry, The University of Rochester, Rochester, New York

As previously reported,* skim milk fortified with vitamin D concentrates exhibits an apparent antirachitic activity 2 to 3 times greater than that expected from the vitamin added. The rôle of phosphorus as a possible cause of this enhancement has been tested. Graded doses of crystalline vitamin D_2, with and without varying amounts of phosphorus (as sodium glycerophosphate), have been fed to over 400 rachitic rats and healing estimated on the 10th experimental day by the radiographic technique.† The vitamin, in propylene glycol solution, was fed daily (in addition to the basal diet) in 4 cc. of 5 per cent gelatin solution containing glycerophosphate. Tests have shown that gelatin is necessary as a protective colloid to prevent loss of vitamin from water solution.

---

* Melnick, D., Cowgill, G. R., and Burack, E., *J. Exp. Med.*, **64**, 877, 895 (1936). We are indebted to Dr. G. R. Cowgill and Dr. D. Melnick for making these proteins available for study.

* Morgareidge, K., and O'Brien, B., *J. Nutrition*, **16**, 395 (1938).

When vitamin D is fed thus, without phosphorus, quantitative recovery of potency results. Amounts fed plotted against observed healing produced by equivalent levels of international standard give a straight line. Feeding 1, 2, and 4 mg. of phosphorus per day plus vitamin D yields corresponding increases in observed healing. Approximately stated, equivalent healing is proportional to the product of the vitamin by the phosphorus fed. More precisely, total antirachitic activity (A) is $A = (1 + k\mathrm{P})\, D$ where P is in mg. and D, international units per day. Constant k equals 0.58 in our colony. Preliminary evidence indicates that all types of phosphorus compounds are not equally effective.

The Production of Anemia by Cholesterol Feeding.

By Ruth Okey and Vera Greaves. *From the Division of Home Economics, College of Agriculture, University of California, Berkeley*

Guinea pigs fed an adequate "synthetic" diet containing 1 per cent cholesterol have developed in addition to the characteristic enlarged and fatty livers a profound anemia and, after 8 to 9 weeks on the diet, spleens of 10 to 12 times the normal weight. Controls fed the same diet without the cholesterol have remained normal throughout the experiment.

Ester cholesterol of liver has averaged in twenty animals fed cholesterol 4.6 per cent, while fatty acid averaged 23.7 per cent and lecithin 2.5 per cent, free cholesterol 0.79 per cent. Control figures were, ester cholesterol 0.20 per cent, fatty acid 6.0 per cent, lecithin 3.7 per cent, and free cholesterol 0.34 per cent.

Free and ester cholesterol figures for blood were approximately twice as high in the cholesterol-fed as in the control animals. There are also significant increases in free cholesterol of spleen, heart, and lungs, with lowered lecithin values in these tissues.

---

† O'Brien, B., and Morgareidge, K., *J. Nutrition*, **16**, 91 (1938).

Histological examination has shown breakdown of red corpuscles and liver cells and necrotic areas in the spleen.

The net effect is a decrease in the lecithin to free cholesterol ratio in the tissues in which cellular destruction has been demonstrated. This suggests that maintenance of this ratio within a definite range may be a necessary condition for normal nutrition.

The Relation of Dietary Protein to Hemoglobin Formation in the Rat.

By James M. Orten and Aline Underhill Orten. *From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*

The relation of dietary protein to the normal blood picture of the rat is being investigated. Animals at weaning (21 days) were fed a synthetic diet adequate except for a low level of protein of excellent quality (lactalbumin). Special attention was given to the vitamin supplements, particularly the vitamin B complex, in order to assure the daily consumption of an abundance of all the known factors. At 100 days of age the animals were in excellent condition, although increase in body weight had been almost entirely inhibited. An examination of the blood, however, showed the existence of a typical hypochromic anemia characterized by a nearly normal red cell count, a low hemoglobin value, and an increased number of reticulocytes. Control rats receiving exactly the same quantities of calories and of all of the dietary constituents except the amount of protein (increased at the expense of carbohydrate) showed a normal blood picture. Furthermore, the feeding of an increased amount of protein (again at the expense of carbohydrate), with no increase in calories or inorganic matter, promptly corrected the anemia. Thus, the anemia is clearly referable to the protein intake. Additional experiments designed to determine the possible effect of the administration of individual amino acids, including glycine, leucine, tryptophane, histidine, cystine, and methionine, and certain other substances, will be discussed.

The Influence of the Melting Points of Fats on Their Utilization by Herbivora.

By Henry Paul and C. M. McCay. *From the Laboratory of Animal Nutrition, Cornell University, Ithaca*

Previous studies have proved that guinea pigs cannot utilize a hard fat as efficiently as a liquid fat. However, analysis showed that this animal could readily digest or split the high melting fat into fatty acids. The poor utilization of the hard fat was considered a result of the guinea pig's inability to absorb to any great degree the higher melting fatty acids.

Experiments were conducted to determine the difference in utilization of two fatty acids having similar composition and saturation but widely different melting points. Liquid oleic acid (m. p. 14–16°; iodine number 90) and its solid crystalline isomer, elaidic acid (m. p. 44°; iodine number 90) were the two fatty acids chosen as meeting these requirements. The elaidic acid was prepared by bubbling oxides of nitrogen through oleic acid. Four recrystallizations of the elaidic acid yielded pure snow-white crystals of the fatty acid.

Rats were able to utilize efficiently the same amount (95 per cent) of either fatty acid. Guinea pigs were able to use 95 per cent of the liquid oleic acid, but used only 55 per cent of the solid elaidic acid.

In pilot studies rabbits and a sheep were able to use to the same degree both a high melting fat (Crisco) and a low melting fat (Wesson oil).

The observation was made that castor oil at a 6 per cent level in the diet was well utilized by sheep, guinea pigs, and rabbits without apparent ill effect. No purgative action resulted from its use.

The Prevalence and Importance of Hypovitaminosis A. By L. B. Pett. *From the Department of Biochemistry, University of Alberta, Edmonton, Canada*

Slight deficiencies of vitamin A, as indicated by adaptation tests, are claimed to be wide-spread. This claim is contested, since convincing evidence, even of the relation of these tests to vitamin A, requires larger groups than yet reported.

A rapid testing apparatus was devised in which an average test takes less than 1 minute, and two or three tests are made within 5 minutes of the subjects entering the room. Confusion from afterglow is avoided, guessing is eliminated (common in children), and the room need not be dark.

Treatment of 269 people having more than 10 seconds recovery time (deficient) with vitamin A brought response in all but two cases, ending in a time of 10 seconds or less (normal). Large amounts of vitamin did not affect people already in the normal range (3 to 10 seconds). The recovery time of a normal person on a vitamin A-deficient diet lengthened from 8 to 27 seconds, when nyctalopia, asialia, and other symptoms were present.

A survey of 1000 university students showed 52 per cent normal, 24 per cent border line, and 24 per cent definitely deficient in vitamin A. Of the normals 54 per cent had a cold within a month, while of the rest 70 per cent had a cold. Similarly dry skin, itchiness, scanty saliva, and dry conjunctiva were several times commoner in the deficient group. Tests in connection with various diseases suggest that hypovitaminosis A is a factor in many conditions involving epithelial tissues.

Over 1300 people have been tested and no relation found between the test and sex, age, color of eyes, wearing of glasses, or other factors. Every deficient case had a low vitamin A intake.

Sulfate and Sucrose Clearances in Normal Men. By MARSCHELLE H. POWER, ARNOLDUS GOUDSMIT, JR., AND NORMAN M. KEITH. *From the Division of Biochemistry and the Division of Medicine, The Mayo Foundation, Rochester, Minnesota*

Solutions containing sodium sulfate and sucrose were injected intravenously in amounts sufficient to attain concentrations in the serum ranging from 70 to 90 mg. per cent of SO_4 and 85 to 170 mg. per cent of sucrose. Initial blood samples were taken 30 to 50 minutes after the cessation of injection. In the succeeding 2 hours the clearances of each of these substances, based on consecutive collections of urine at intervals of 30 minutes, did not change appreciably as their concentrations in the serum decreased about 50 per cent. The clearance of sulfate, which is only 25 to 40 per cent of that of sucrose at endogenous levels of the serum sulfate, increased markedly at the higher levels, and attained values representing 89, 101, and 108 per cent of the sucrose clearance in different individuals. These results are consistent with the view that sulfate is excreted by glomerular filtration with subsequent reabsorption which at high concentration of SO_4 in the serum may be less than the reabsorption of sucrose. Experiments with dogs* in which the clearance of SO_4 at high concentrations in the serum approached that of creatinine—a substance widely accepted as a measure of the rate of glomerular filtration in the dog—lead to a similar view of the mechanism of excretion of SO_4. The volumes of distribution of sucrose and sulfate were found to be practically identical.

---

* Goudsmit, A., Jr., Power, M. H., and Bollman, J. L., *Am. J. Physiol.*, in press.

The Determination of the Vitamin A Value of Tissue of Low Oil Content by the Carr-Price Reaction and the Percentage of Oil on the Same Sample. By L. I. Pugsley. *From the Fisheries Experimental Station, Prince Rupert, Canada*

The determination of the vitamin A value of tissue of low oil content (*e.g.* halibut liver and viscera) presents a number of difficulties owing to the instability of vitamin A. In order to overcome some of these, a method has been worked out whereby a small sample (10 gm.) of raw minced tissue is ground with anhydrous sodium sulfate (30 gm.), extracted with chloroform (250 cc.), made up to a definite volume, and a suitable aliquot of the chloroform extract taken for the determination of vitamin A by the Carr-Price reaction, in the Evelyn photoelectric colorimeter. The chloroform is distilled off from the remaining portion, and the oil residue dried in a vacuum oven and weighed.

The chloroform must be added immediately after the tissue is ground with the sodium sulfate; otherwise loss of vitamin A occurs. Better extraction was obtained with raw tissue than with cooked, the latter forming a hard cake which adhered to the extraction flask. No loss of vitamin A was detected when the tissue stood in the presence of the chloroform for 6 hours. However, when the mixture was allowed to stand for 15 hours, a definite decrease in the value occurred.

The same recovery of vitamin A was obtained by this method as by alkali digestion of the tissue and ether extraction of the unsaponifiable matter. The yield of oil also compared favorably with that obtained by ethyl ether extraction.

The Concentration of Prothrombin in Infant Blood. By Armand J. Quick. *From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

Experimentally and clinically it has been demonstrated that an adequate intake and absorption of vitamin K are essential for the maintenance of a normal concentration of prothrombin in the blood. Since the writer has shown that a prothrombin deficiency can be detected in chicks 4 days after the removal of vitamin K from the diet, the possibility of a similar deficiency in infants must be considered, especially in view of the fact that Brinkhous, Smith, and Warner* have reported that the concentration of prothrombin in the blood of infants below the age of 2 months and of cord blood is less than 50 per cent of normal. By using the method developed by the writer for the quantitative determination of prothrombin, it was found that the concentration of prothrombin in infant blood, even of babies 7 and 8 days old, was the same as that of the blood of normal adults. The prothrombin of cord blood was also found approximately normal, but certain plasmas obtained from such bloods had a slight antithrombic action. In an infant 6 months old with congenital atresia of the common bile duct, a normal prothrombin was found. Although there was no evidence of any bile having ever entered the intestines up to this time, no deficiency of vitamin K, as indicated by the normal level of prothrombin, could be demonstrated. It appears therefore that vitamin K deficiency in infants is uncommon.

The Antipneumococcic Effect of Sulfapyridine and Related Compounds in Mice. By George W. Raiziss, M. Severac, J. C. Moetsch, and Leroy W. Clemence. *From the Graduate School of Medicine, University of Pennsylvania, and the Dermatological Research Laboratories, Philadelphia*

Whitby and other English investigators reported good therapeutic effects obtained by the use of sulfanilylaminopyridine (sulfapyridine) in the treatment of experimental pneumococcic infections in mice. Favorable clinical results in human pneumonia were also reported.

Mice were infected by us with virulent strains of pneumococcus Types I, II, and III and treated by the oral administration of sulfapyridine, sulfanilamide, and diaminodiphenylsulfone. The same drugs were tested in experimental β-hemolytic streptococcus infection in mice. The comparative therapeutic results, as well as data concerning toxicity, are being presented.

---

* Brinkhous, K. M., Smith, H. P., and Warner, E. D., *Am. J. Med. Sc.*, **193**, 475 (1937).

The Effects of Insulin on Serum Lipids and Choline Esterase in Schizophrenic Subjects. By Lowell O. Randall. *From the Research Service, Worcester State Hospital, Worcester*

In contrast to the usual lowering effect of insulin on the lipemia of diabetes and on the lipid level of normal animals we found an immediate as well as a prolonged rise in serum lipids during the insulin shock treatment of schizophrenic subjects and a concomitant increase in the choline esterase activity. Bloor's oxidimetric methods were used for the determination of phospholipid, total and free cholesterol, and total lipid and Ammon's* gasometric method for the choline esterase activity of serum. Two blood samples, 1 week apart, were taken before and two after treatment. During the 3rd and 7th weeks of treatment, samples were taken before insulin was given, during coma, and 1 hour after sugar administration. In twelve patients, during the period of coma, there was a statistically significant rise in all the lipid fractions and in the choline esterase activity and a smaller increase in red cell volume. This latter accounted for about 20 per cent of the increase in the lipid and esterase. These values returned toward the control level after administration of sugar. In seventeen patients, a prolonged effect of insulin was observed. There was a significant rise in serum lipids and choline esterase activity which remained above the initial level for at least 2 weeks after cessation of treatment. These effects are believed to have been due to the stimulation of the sympathetico-adrenal system by insulin.

Serum and Urinary Phosphorus Changes in Man after Olive Oil Ingestion. By Raymond Reiser and Frederick M. Hanes. *From the Departments of Medicine and Biochemistry, Duke University School of Medicine, Durham, North Carolina*

Data in the literature on phosphorus changes in the serum and urine after fat ingestion are meager and conflicting. In the present study 50 gm. of olive oil were fed to a number of normal persons in the fasting state at 8 a.m. and the serum inorganic phosphorus and rate of urinary phosphorus excretion determined 0.5, 1, 2, 3, and 4 hours afterwards. The subjects remained in bed during the entire course of the experiments.

---

* Ammon, R., *Arch. ges. Physiol.*, **233**, 486 (1933).

In eight of nine cases the serum inorganic phosphorus was reduced, reaching a minimum 1 hour after the meal. The smallest decrease was 0.18 and the largest 0.46 mg. per cent, the average being 0.32 mg. per cent or 8.2 per cent of the fasting value of 3.9 mg. per cent. 3 hours after the meal the serum phosphorus had returned to the fasting level.

In each of ten cases there was a very pronounced drop in the rate of urinary phosphorus excretion expressed as mg. of P excreted per hour. The minimum excretion was in the period between 0.5 and 1 hour after the ingestion of the oil, the average decrease in rate being 70 per cent of the fasting value. The smallest decrease was 50 per cent and the largest 90 per cent. The rate of excretion rapidly increased but in only four cases had it returned to or exceeded the fasting level at the end of the experiment.

These changes are considered greater than can be accounted for on the basis of diurnal variation.

Further Studies on Ammonia Metabolism. By D. Rittenberg, Albert S. Keston, and Samuel Graff. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

It has been shown that isotopic ammonia given to rats under exceptional conditions may be used for the formation of amino acids. It has now been found that this also occurs under normal conditions. To the stock diet of adult rats was added a small amount of isotopic ammonia, and a number of pure amino acids were isolated from the proteins of the liver and intestinal tract. The presence of isotope in the amino acids shows that dietary ammonia was involved in their formation.

The uptake of isotopic nitrogen into the guanido group of arginine has been investigated more closely.

After feeding isotopic ammonia the urinary ammonia contains only traces of isotope, while the concentration of it in the urinary urea is high. Feeding of isotopic amino acids leads to the reverse condition; high concentration in the urinary ammonia, low concentration in the urea.

The Determination of Cholesterol. By Anton R. Rose, Fred Schattner, and William G. Exton. *From the Laboratory and Longevity Service of The Prudential Insurance Company of America, Newark*

As the green color of the Liebermann-Burchard reaction is unsatisfactory, the determination of cholesterol in a system of lipid partition developed by us* utilizes the red color of the Tschugaeff reaction.

Add 0.1 cc. of blood to 5 cc. of equal parts of alcohol and acetone; after standing in a bath 5 minutes at 80° centrifuge. For total cholesterol saponify 4 cc. of the clear liquid with 1 drop of concentrated sodium hydroxide. Then separate the cholesterol as digitonide as usual. After washing with alcohol-ether and drying at 40° develop the red color by adding 3 cc. of zinc chloride (38 gm. in 100 cc. of glacial acetic acid), then 2 cc. of acetyl chloride. Immerse immediately in water at 55° and stir briskly until digitonide is completely dissolved. Then stopper with cork protected by tin-foil and stand in the bath 15 minutes longer.

The red color is directly proportional to the concentration of cholesterol and constant more than an hour. It is best measured in the electroscopometer but can also be measured with the usual simultaneous standards in the Duboscq type of apparatus.

The Amino Acids of Tobacco Mosaic Virus Protein. By A. Frank Ross and W. M. Stanley. *From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*

The hydrolytic products of chemically isolated tobacco mosaic virus protein were subjected to a systematic fractionation involving the separation of the basic amino acids as their silver salts, precipitation of the barium salts of the dicarboxylic amino acids by alcohol, and the preparation of the copper salts of the remaining monoamino acids. Individual amino acids were crystallized from the various fractions or were obtained in the form of suitable derivatives. The tobacco mosaic virus protein appears to contain no histidine, less than 0.5 per cent lysine, 8.8 per cent arginine, 5.1 per cent glutamic acid, 4 per cent aspartic acid, 4.2 per cent leucine, and 3.9 per cent valine. Colorimetric methods of estimation indicated the presence of 2 per cent tryptophane, 3.8 per cent tyrosine, and 0.6 per cent cysteine. Methionine was found to be present in traces. Use of Bergmann's reagents indicated a proline content of 4.6 per cent but failed to establish the presence of alanine or glycine. Data on other amino acids are given. As a check on the purity of the chemically prepared virus protein, a smaller quantity of the protein was isolated by means of an ultracentrifuge and used for the estimation of several of the amino acids. No differences were found in the amino acid content of the two preparations.

---

* Rose, A. R., Schattner, F., and Exton, W. G., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **119**, p. lxxxiv (1937).

The Decomposition of Cysteine in Aqueous Solution. By JOSEPH I. ROUTH. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Cysteine hydrochloride (1.3 gm.) dissolved in distilled water was brought to approximately pH 7.0 by the addition of the theoretical amount of sodium hydroxide and the solution was adjusted to a total volume of 1 liter. On boiling the solution, the formation of cystine and hydrogen sulfide was observed. No elementary sulfur could be detected as contrasted to the formation of appreciable amounts of elementary sulfur when cystine was decomposed under similar conditions. Also in contrast to the work recently described on cystine the breakdown of cysteine was more rapid and extensive in air than in an atmosphere of nitrogen. In all but the longest periods of boiling (48 hours) in air approximately 100 per cent of the original sulfur could be accounted for as cysteine, cystine, hydrogen sulfide, and a small amount of an unknown form associated with increased acidity. During the decomposition of cysteine there was a slight loss of nitrogen which increased with the time of boiling and was greater in air than in nitrogen. This is in contrast to the behavior of cystine, whose nitrogen remained unchanged.

The Effect of Different Levels of Vitamin B$_1$ and Iron on the Retention of Iron and the Fat Content of Normal Young Rats.

BY FREDERIC W. SCHLUTZ AND HELEN OLDHAM. *From the Department of Pediatrics, the University of Chicago, Chicago*

Litter mate rats were given the same diets but varying amounts of vitamin B$_1$ hydrochloride and iron. The iron content of their bodies was determined at 1, 2, and 3 weeks and retentions were calculated; as a control a third litter mate was used which had been sacrificed and analyzed for iron at the beginning of the experiment.

The level of vitamin B$_1$ intake did not influence the retention of iron at the levels studied. The optimum daily iron intake for rats of this age as judged by the retention of iron and by the iron content of the bodies is not more than 0.30 mg. Intakes above this level failed to give an increase in storage. An experimental period of 7 days was found to be of sufficient length to judge retention of iron.

When isocaloric intakes were given, animals receiving 39 micrograms of vitamin B$_1$ daily gained on an average 1.1 gm. per week more than their litter mates who were receiving 9 micrograms of the vitamin. This difference was found to be significant. Of the increased gain, 90 per cent was found to be accounted for by increases in the fat and water content of the bodies.

The Therapeutic Properties of 2-Sulfanilamidopyridine in Experimental Pneumococcus Infections.

BY L. H. SCHMIDT. *From the Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati*

This report concerns the therapeutic effectiveness of 2-sulfanilamidopyridine in experimental infections with Types I, II, III, IV, V, VI, A and B, VII, VIII, XI, XX, XXII, XXIV, XXVII, and XXIX pneumococci. White mice were infected intraperitoneally with 100 to 1000 lethal doses of a given type pneumococcus. 2 hours after infection they were treated with a certain amount of sulfanilamidopyridine; this treatment was repeated at varying intervals for periods of 6 to 10 days. If the mice survived 30 days after infection and had negative blood cultures at that time, they were considered cured.

The results show that the effectiveness of sulfanilamidopyridine therapy is not equal in infections with all types of pneumococci, even though these pneumococci are of similar virulence. As judged by the amount of drug and duration of treatment required, sulfanilamidopyridine had its greatest effect in infections with Types I and VII pneumococci, next in Types IV, V, VI, A and B, XI, XXII, and XXIX, and next in Types XX, XXIV, and XXVII, having a marked curative action in infections with all these types. In infections with Types II, III, and VIII the drug had little curative action, although it did prolong life.

Low Phosphorus Diets and Urinary Lithiasis. BY H. SCHNEIDER AND H. STEENBOCK. *From the Department of Biochemistry, University of Wisconsin, Madison*

Urinary calculi have been produced consistently in young rats on a purified low phosphorus diet (P = 0.04 per cent, Ca = 0.57 per cent). The diet contained ample amounts of vitamins A and D and was complete in all other respects. No calculi have ever been observed in rats on this diet when an adequate supply of phosphorus was furnished by a normal salt mixture. On the low phosphorus diet single calculi have been observed as early as the 7th week and after 20 weeks pathological changes in the urinary tract varied from a unilateral calculus to extensive involvements with hydronephrosis and multiple vesical calculi.

Analysis of the calculi revealed that they were composed of practically pure calcium citrate. Phosphate was present only in traces. This is in marked contrast to reported analyses of human calculi. Our own analysis of several human urinary calculi has revealed citric acid contents varying from 0.0 to 1.5 per cent. It is suggested that calcium citrate may play a rôle in the formation of the primary nidus upon which other urinary salts precipitate to form the calculus.

The urine of the rats on the low phosphorus diet was characterized by a high pH, low P, and high Ca content. The urinary citric acid output of the experimental rats was higher than that of controls on the same diet but receiving a normal salt mixture. Experiments are being conducted to determine whether the increased citric acid is due to changes in metabolism referable to the deprivation of phosphorus.

Amino Acid Metabolism with Isotopes As Indicators. By Rudolf Schoenheimer, S. Ratner, and D. Rittenberg. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

Leucine containing carbon-bound deuterium and N^{15} has been resolved into its optical enantiomorphs. The natural form has been added to the stock diet of full grown rats for 3 days. In agreement with previous experiments on the metabolism of tyrosine only a fraction of the nitrogen isotope was excreted, while the greater part was recovered from the proteins of the animals. The distribution of the isotope in different fractions and in pure amino acids isolated from the proteins has been investigated.

Glycine was injected into rats with and without simultaneous addition of benzoic acid. Glycine was then isolated from the proteins of different tissues. The isotope content of these samples was determined.

The Effect of Deficiencies in Copper and Iron on Oxidation Catalysts of Rat Tissues. By M. O. Schultze. *From the Department of Chemistry, University of Pittsburgh, Pittsburgh*

Rats were fed from weaning on whole milk supplemented with Mn and either Fe or Cu or Fe plus Cu. The oxidation of ascorbic acid with homogenized liver from these animals was determined. The rate of ascorbic acid oxidation during 2 hours was the same with livers of animals getting 0.5 mg. of Fe per day plus 0.5 mg. of Cu or 0.05 mg. of Cu per day respectively. With livers from rats getting no Cu and suffering from severe deficiency (hemoglobin 2 to 3 gm. per 100 cc.), the rate of ascorbic acid oxidation was even greater. Hence, copper can accumulate in the liver in a form catalytically inactive towards oxidation of ascorbic acid.

With homogenized liver and heart from the same animals, cytochrome oxidase activity was determined manometrically in a buffered system containing cytochrome C, hydroquinone, semicarbazide, and tissue (about 3 mg., dry). The cytochrome oxidase activity of the tissues of the animals getting 0.5 mg. of Fe per day plus 0.5 mg. or 0.05 mg. of Cu per day was the same (Q_{O_2} of liver 98, of heart 140). In severe copper deficiency it was greatly decreased (Q_{O_2} of liver 19, of heart 55). This decrease, particularly in the liver, is greater than could be accounted for by the hypertrophy of the organs studied.

Similar determinations on tissues from rats suffering from iron deficiency will be reported.

The Relation of Vitamin C to the Metabolism of the Melanin Pigment Precursors, Tyrosine and Dihydroxyphenylalanine.

By Robert Ridgely Sealock, Benjamin Ziegler, and Robert L. Driver. *From the Department of Vital Economics, The University of Rochester, Rochester, New York*

The demonstration by various investigators of the relationship of ascorbic acid to the pigmentation of Addison's disease and to the production *in vitro* of melanin by the enzymes tyrosinase and dopase suggested that the feeding of the melanin precursors, tyrosine and dihydroxyphenylalanine, to animals susceptible to vitamin C deficiency should furnish a means of studying some further aspects of the relationship. Guinea pigs have been fed daily supplements of these precursors with and without the maintenance level of vitamin C. By comparison with suitable controls there has been demonstrated, by the usual criteria of deficiency, an increased requirement for ascorbic acid when the amino acids are being metabolized. Since albino animals are incapable of synthesizing melanin, similar experiments have been performed with albino guinea pigs. The results indicate that these animals do not exhibit the same increased requirement for ascorbic acid as do the pigmented ones.

The Effect of Cystamine on the Albino Rat.

By W. H. Sebrell and Floyd Shelton Daft. *From the National Institute of Health, Washington*

Cystamine was prepared according to the Gabriel synthesis. A portion was recrystallized to a constant melting point of approximately 215° and a mixed melting point taken with a carefully analyzed sample from another laboratory. Young albino rats weighing from 53 to 57 gm., with litter mate controls, were fed 0.5 per cent of both crude and recrystallized cystamine in a 4 per cent casein diet. All cystamine-fed rats died in from 12 to 19 days and had unusually brittle femurs, easily detachable epiphyses, and other bone changes, which in some instances were visible on x-ray examination.

Two lots of young albino rats on an adequate stock diet, with litter mate controls, weighing from 44 to 62 gm., were given 1 and 0.5 mg. of cystamine per gm. of body weight daily in aqueous solution by stomach tube. All rats receiving 1 mg. per gm. of body weight daily were dead after not more than two doses and those receiving 0.5 mg. per gm. of body weight daily were dead or moribund after not more than seven doses. All showed hemorrhagic changes around the knee joints.

Another lot of young adult albino rats on an adequate stock diet, with suitable litter mate controls, weighing 152 to 188 gm., also were given 1 mg. of cystamine daily by stomach tube. All the rats fed cystamine were dead or moribund after not more than six doses.

The Effect of Lyophilizing and Other Methods of Preserving Tuberculin Protein.

By Florence B. Seibert and Emma H. DuFour. *From the Henry Phipps Institute, University of Pennsylvania, Philadelphia*

Preservation with 0.5 per cent phenol or 1:10,000 merthiolate of tuberculin culture filtrate previously caused very little change in the ultracentrifuge velocity sedimentation characteristics. Moreover, preservation with 0.5 per cent phenol for at least 3 years of a 1 per cent solution of tuberculin protein, isolated as the purified protein derivative, caused no decrease in potency.

Preservation in dried form, however, should be preferable. It is known that dried purified protein derivative will retain its potency indefinitely, but is then not completely water-soluble. Previous study of a more labile fraction showed that it could be either frozen at −8° to −10° or dried *in vacuo* with its buffer at pH 8 without effect on its sedimentation characteristics or solubility. Therefore, the "lyophile" process of Flosdorf and Mudd was applied to a highly purified protein derivative preparation in the presence of phosphate buffer of pH 7.3. It was found to be completely soluble in water and, when examined in the Tiselius electrophoresis apparatus, showed a single protein component, as evidenced by one persisting sharp "schlierien" band. Previous experiments have shown that with denaturation of the tuberculin protein the band tends to become diffuse and even splits into two. Furthermore, a purified protein derivative solution diluted to the usual diagnostic doses (0.0002 and 0.05 mg. per cc.), lyophilized, and supplied to us by Dr. John Reichel of Sharp and Dohme for clinical tests showed no loss in potency. Nor did a change in potency occur in a 0.1 per cent purified protein derivative lyophilized and then incubated for 21 to 28 days at 37.5°.

The Rôle of Cations in Carbohydrate Oxidation. By Fay Sheppard and Mark R. Everett. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

Oxidation of carbohydrates is inhibited by hydrogen ions, but other cations accelerate it. We have completed studies of the effects of thirty-seven inorganic cations, both as acetates and as carbonates, basic carbonates, or hydroxides, having previously demonstrated that the last three have approximately equal accelerating influences upon carbohydrate oxidation. 4 equivalents of each salt were added to 1 per cent *d*-gluconolactone solutions. Acetate mixtures were allowed to react with bromine 48 hours, and carbonate mixtures 30 minutes, at 25°. Average keturonic acid production was as follows: iron, zirconium, and uranyl, 40 to 45 per cent; aluminum, copper, and silver, 30 to 35 per cent; calcium, cerium, and samarium, 23 per cent; lithium and thallium, 20 per cent; barium, indium, potassium, manganese, sodium, and lead, 16 per cent; strontium, yttrium, gold, mercury, and neodymium, 13 per cent; cobalt and magnesium, 10 per cent; while thorium, cadmium, chromium, lanthanum, bismuth, nickel, praseodymium, erbium, beryllium, tantalum, zinc, and columbium gave smaller amounts, decreasing in the order named. The results reported for bismuth, beryllium, gold, indium, lanthanum, neodymium, praseodymium, and samarium are minimal, because of partial insolubility of the salts employed. Keturonic acid production in carbonate mixtures was near equilibrium at 30 minutes, little difference being encountered at 1 to 2 hours. Iron, zirconium, uranyl, aluminum, silver, and copper are the most effective cations for carbohydrate oxidation. They function best when introduced as salts of poorly dissociated acids. Our interpretation of the rôle of inorganic substances in carbohydrate oxidation awaits completion of similar studies with anions.

Magnesium Balance Studies with Infants.

By Carroll F. Shukers, Elizabeth M. Knott, and Frederic W. Schlutz. *From the Department of Pediatrics, the University of Chicago, Chicago*

Since relatively few magnesium balance studies on children have been reported in the literature, magnesium retentions have been determined for seven male infants 2 to 6 months of age. During 57 metabolic periods, of 5 days each, the magnesium intake varied from 11.8 to 39.8 mg. per kilo of body weight per day, with retentions varying from slightly negative balances to 16.0 mg. per kilo. 58 per cent of the retentions were within the range of 0.0 to 6.0 mg. per kilo. When individual infants were considered, there was a trend toward higher retentions with increases in intake, but a much more definite correlation existed between the level of intake and the amount of magnesium excreted in the urine. The ratio of urinary to fecal excretion was approximately 1:8 to 12 as contrasted to ratios of 1:2 which have been reported for preschool children.

The type of added carbohydrate, whether honey or corn syrup, and the level of vitamin B_1 in the diet had no effect on the magnitude of retentions. Increases in intake of a vitamin D solution also had no effect upon retentions, but an increase in cod liver oil was correlated with somewhat higher retentions in one infant. Powdered protein milk, as contrasted to Dryco or evaporated milk, had a tendency to improve absorption of the magnesium, with resulting increases in urinary excretion and in retentions. There was a slight correlation between calcium and magnesium retentions, indicating probably that both had been affected by the same factors.

The Rate of Turnover of Lecithins and Cephalins.

By Robert Gordon Sinclair. *From the Department of Biochemistry, Queen's University, Kingston, Canada.*

Except for the specific activity of the cephalins in the clotting of blood, no information is as yet at hand as to the precise physiological functions of each of the three groups of phospholipids, the lecithins, cephalins, and sphingomyelins. In recent years the use of elaidic acid or of radioactive phosphorus has clearly shown that in the liver but not in the muscle a considerable part of the phospholipids, taken as a whole, are intermediaries in fat metabolism. On various grounds it appeared possible that these metabolic phospholipids might be entirely of the lecithin type.

To study this point, the phospholipids of both liver and muscle were fractionated into cephalins and lecithins by means of absolute alcohol. The degree of separation was checked by choline determination. It was found that the uptake of elaidic acid, in terms of the percentage of total fatty acids, was somewhat greater in the lecithins than in the cephalins of both liver and muscle.

By determining the rate of uptake of elaidic acid it was found that the rate of turnover of the lecithins in the liver was definitely more rapid than that of the cephalins. However, the rate of turnover of the cephalins was so rapid that it appears likely that they as well as the lecithins function as intermediaries in fat metabolism.

Stability of the Vitamin B Complex to Sulfur Dioxide and Autoclaving. BY HENRY A. SMITH AND MILDRED K. DIMICK. *From the Chemical Laboratory of the Galen Company, and the Biological Laboratory of The Vitab Corporation, Berkeley, California*

A rice bran concentrate containing 50 international standard units of thiamine, 10 micrograms of riboflavin, 150 micrograms of Factor 1, and a filtrate factor value of approximately 28 units per cc. was treated with sulfur dioxide. The thiamine (vitamin B_1) was completely destroyed. The riboflavin was partially destroyed; the Factor 1 (vitamin B_6) and the chick antidermatitis factor suffered little or no destruction. Sulfur dioxide under the same conditions destroys little or no vitamin B_1 in its pure form or as a barium hydroxide eluate from a fullers' earth adsorbate of a rice bran extract. Sodium sulfite, on the other hand, when adjusted to pH 5 with sulfur dioxide destroys vitamin B_1 both in its pure state and in the form of an eluate.

Autoclaving almost completely destroys the vitamin B_1 in yeast, but vitamin B_1 in its pure form is quite stable. The chick antidermatitis vitamin is partially destroyed by autoclaving, both in yeast and rice bran concentrate. The rice bran concentrate treated with sulfur dioxide is a good supplement for the Chase and Sherman vitamin B-free diet, as it provides those members of the vitamin B complex which are often not supplied by the autoclaved yeast.

Some Properties of a Hemolysin Produced by Group A β-Hemolytic Streptococci.

By C. V. Smythe and T. N. Harris. *From the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia*

If Group A β-hemolytic streptococci are cultivated on a meat infusion broth and the organisms are then removed by centrifugation or filtration, the broth will hemolyze red blood cells. It is known that exposure of the broth to oxygen causes a slow disappearance of the hemolytic activity and that the addition of sodium hydrosulfite ($Na_2S_2O_4$) to the oxygen-inactivated broth promptly restores the hemolytic activity. Very little has been known of the chemical nature of this hemolysin.

The work on which the present report is based shows that the agent responsible for the hemolytic activity does not dialyze through a cellophane membrane, is precipitated by ammonium sulfate, and is inactivated by pepsin, trypsin, and papain. The oxygen-inactivated form of the hemolysin has been partially purified to a stable, dry powder. This powder dissolved in saline is rendered hemolytic by treatment with sodium hydrosulfite, sodium bisulfite, cysteine, hydrogen sulfide, or potassium cyanide. It is not rendered hemolytic by pyrophosphate or palladium asbestos and hydrogen. Ascorbic acid or ascorbic acid plus iodide makes it only very slightly hemolytic. After any of these unsuccessful treatments the material may still be made fully hemolytic by any of the effective reagents. The partially purified activated material is inactivated by oxygen, hydrogen peroxide, iodine, potassium ferricyanide, iodoacetamide, phenylmercuric chloride, or cuprous oxide. In each case the inactivated material can be at least partially reactivated by the above activating reagents.

Properties of a New Growth Factor for Lactic Acid Bacteria.

By E. E. Snell and W. H. Peterson. *From the Departments of Biochemistry and Agricultural Bacteriology, University of Wisconsin, Madison*

Studies from this laboratory have shown that *Lactobacillus casei* and certain related organisms require riboflavin, pantothenic acid, and nicotinic acid for growth on media lacking these substances. Quantitative assays for riboflavin and pantothenic acid have been developed utilizing these requirements.

When the culture of *Lactobacillus casei* is attempted in a hydrolyzed casein medium containing these substances, little or no growth occurred until extracts of yeast, liver, etc., were added. By norit adsorption from acid solution this growth essential was split into two fractions, both of which were required for proper growth.

The portion readily adsorbed on norit was eluted by pyridine-alcohol mixtures, and was further investigated. It is fairly stable to both acid and alkali, but is destroyed by prolonged hydrolysis with either agent. Fractions containing it are insoluble in 95 per cent alcohol, but it is extracted from solution by butyl alcohol at pH 4.0. It is precipitated by basic lead acetate and is firmly adsorbed by lead sulfide and other metallic sulfides. It forms a water-insoluble picrate, which has not been obtained in crystalline condition. Acetylation inactivates the substance.

The effect of the substance on growth cannot be duplicated by mixtures of any known growth factors. The purest preparations obtained can be detected in concentrations of 0.01 to 0.03 microgram per cc.; maximum stimulation occurs with 0.3 to 0.5 microgram per cc. A potent natural source, yeast extract, similarly tested shows maximum activity in concentrations of 200 to 300 micrograms per cc.

Microestimation of Calcium in One-Tenth Cubic Centimeter of Serum. By Albert E. Sobel and Bernard A. Sobel. *From the Pediatric Research Laboratory and Division of Biochemistry, The Jewish Hospital of Brooklyn, New York*

A simple method for the estimation of calcium in 0.1 cc. of serum was developed. This procedure is a modification of that previously reported from this laboratory,* for the acidimetric microtitration of calcium by a direct titration.

Calcium is precipitated as the oxalate in a specially designed, small centrifuge tube. This is then converted to the carbonate at 475–525°. The carbonate is dissolved in a hot solution of 10 per cent boric acid. The solution is titrated by a capillary microburette to the pH of a pure boric acid solution. The burette is regulated by a screw arrangement and its tip dips into the solution during titration. The results compare favorably with those by previous methods, where 2.0 cc. of serum are employed.

---

* Sobel, A. E., and Sklersky, S., *J. Biol. Chem.*, **122**, 665 (1937-38).

The technique of the new procedure is simple and readily adaptable to the routine work of a pediatric department where the amount of blood available for an analysis is often limited.

Influence of Dietary Phosphorus, Calcium, and Vitamin D in Experimental Lead Poisoning.

By Albert E. Sobel, Henry Yuska, David D. Petrovsky, and Benjamin Kramer. *From the Pediatric Research Laboratory of The Jewish Hospital of Brooklyn, New York*

Experiments were designed to study the influence of phosphorus, calcium, and vitamin D in lead poisoning. A basal diet was devised which contained traces of calcium (0.03 per cent) and small amounts of phosphorus (0.25 per cent) to which 1 per cent basic lead carbonate was added (0.82 per cent Pb). This basal diet was used without other additions as a low calcium, low phosphorus diet. $CaCO_3$ (2.5 per cent) was added in one case to the basal diet to produce a high calcium, low phosphorus diet; and Na_2HPO_4 (2.75 per cent) in another case to produce a high phosphorus, low calcium diet. Each of these three diets was fed to about forty rats 23 to 25 days old (albinos of an original Wistar strain raised on a Bills stock diet). One-half of each group was given 100 international vitamin D units daily after 5 days on the experimental diet. After 28 days the animals were sacrificed and the lead content of the blood and bones determined.

Vitamin D was found to increase the percentage of lead in the bone ash from 2- to 4-fold in all groups. In the low calcium, low phosphorous diet the whole blood lead value was distinctly higher in the vitamin D groups. In the high calcium, low phosphorus group the difference in blood lead values due to vitamin D was not significant. In the high phosphorus, low calcium groups the level of the blood lead was much lower than in either of the previous two groups and the effect of vitamin D could not be reliably measured in view of the very low concentrations of lead in the blood.

In considering the biochemical behavior of lead as influenced by the diet, both the calcium and phosphorus levels as well as the presence or absence of vitamin D must be taken into account, just as in calcification.

A Study of Cholesterol Esterase in Brain and Liver. BY WARREN M. SPERRY AND FLORENCE C. BRAND. *From the Chemical Departments of the Babies Hospital and the New York State Psychiatric Institute and Hospital, and the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

A marked esterification of free cholesterol has been shown to occur during incubation *in vitro* of blood serum from all animal species studied. If the reaction also takes place *in vivo,* there must be a continuous removal from the serum, and presumably hydrolysis, of cholesterol esters by the tissues. In an effort to determine the possible rôle of brain and liver in such processes emulsions of these tissues from rats and mice were prepared in distilled water or buffers and incubated in experiments similar to those employed in the serum studies. In brain emulsions no esterification of free cholesterol occurred. There was also no esterification on incubation of brain slices in other experiments and it is concluded that a "synthesizing" cholesterol esterase is not present in rat or mouse brain. A significant esterification was usually found in rat liver emulsions in distilled water despite the low pH values (4 to 5) which obtained. In the presence of phosphate buffers of varying concentration there was little or no esterification, regardless of the final pH. On incubation of mixtures of rat serum with rat brain or liver emulsions little or no effect of the serum in promoting esterification of tissue-free cholesterol could be demonstrated. On the contrary, there was usually complete inhibition of the normal esterification of the serum and even in some cases, particularly in mixtures with liver emulsions, a tendency toward hydrolysis of cholesterol esters.

x-Ray Studies on Membranes.* BY MONA SPIEGEL-ADOLF AND GEORGE C. HENNY. *From the Department of Colloid Chemistry,*

---

* Aided by a grant from the National Research Council, Committee on Radiation, to M. Spiegel-Adolf.

D. J. McCarthy Foundation, and the Department of Physics, Temple University School of Medicine, Philadelphia

x-Ray diffraction studies were made on various membranes in order to correlate the electric behavior of membranes to their structure and to study the effects of radiant energy on protein diaphragms.

One of us (S.-A.) has studied the polarizability of membranes and devised a method to vary it. In collodion membranes, it was not possible to decide whether the changes in polarizability were based upon variations in the size of the pores or upon changes of the primary particles. x-Ray diffraction studies on nonpolarizable and polarizable collodion membranes indicate a noticeable difference between their respective diffraction patterns: the compression which causes the polarizability seems to call forth a shrinkage of the diameter of one diffraction ring, while the others are unchanged. These findings suggest at least a participation of the primary collodion particles in changes producing polarization and also suggest reasons for changes in certain lines of the diffraction pattern.

Dry gelatin sheets were exposed to a prolonged x-ray irradiation. A control was heated extensively. The x-ray picture of this control revealed a shrinkage of one ring and a diminished intensity of all the rings. Both changes were reversible with hydration. The irradiated sample did not show any changes in the diameters of the diffraction rings, although the intensity of the pattern was even lower than in the heated gelatin; nor could any reversibility of the changes of intensity be detected in a moist condition. These findings confirm previously stated differences in the effects of heat and radiant energy upon proteins.

The Action of Insulin upon Urea Formation and Carbohydrate Synthesis by Liver Slices of Normal and Diabetic Animals.

By William C. Stadie, F. D. W. Lukens, and John A. Zapp, Jr.
From the Department of Research Medicine, and the Cox Institute, University of Pennsylvania, Philadelphia

In normal fasted rats insulin depressed urea formation by liver slices *in vitro* from *dl*-alanine and *l*-alanine. With *d*-alanine no decrease was found. The inhibition of urea formation by insulin was not confined to glycogenic amino acids, contrary to the hypothesis of Bach and Holmes. Consistent effects of insulin on deamination of amino acids were observed only with the non-natural amino acids. Deaminase prepared from kidney was not inhibited by insulin. After intravenous insulin, deamination by liver slices was found to be less than normal.

In normal fasted cats the results were essentially those found in the rat. The diabetic liver slices showed one-third greater deamination than the normal. The effects of insulin, however, were found not to be increased. Houssay cats were like the normals, rather than the diabetic.

No effect of insulin upon carbohydrate synthesis by liver slices was observed. The carbohydrate synthesis by diabetic cat liver slices was not found increased; here, too, insulin was without effect.

Insulin had no significant effect on the respiration of normal or diabetic liver slices.

The Acidic and Basic Properties of Wool Protein. By JACINTO STEINHARDT AND MILTON HARRIS. *From the Research Laboratory of the Textile Foundation at the National Bureau of Standards, Washington*

The extent of combination of wool fibers with acids and bases, in dilute solutions of constant ionic strength, depends on the concentration of salt as well as on the concentration of acid or base. Directly related to this effect is the profound difference in shape of the titration curve when measured in the absence of salt. The slope is steeper, and the equation describing it is a function of $[H^+]^2$ instead of $[H^+]$ in the mass law relation which governs the results at constant ionic strength. The absence of combination at pH 4.7 to 7.0, previously reported, is observed only when salt is absent; in 0.1 M KCl, acid combination begins near pH 6.4 where buffering, due to histidine, appears.

These effects are not accounted for by the usual application of the Donnan equations, nor by the effect of salts on dissociation constants. They are predictable consequences of a progressive adsorption of ions, paralleling the titration curve, brought about by potential differences between fiber and solution which would result if only hydrogen ions combined. Simple equations describe the consequent restriction on combination when ions are furnished only by the acid. With salt present the restriction weakens, and, with high constant salt concentrations, disappears. Thus only at concentrations above 0.1 M do components which can be allocated to specific groups appear in their customary pH ranges. At these concentrations and from pH 1 to 7, the titration curves of wool and egg albumin (with approximately equal ratios of imidazole to free carboxyl groups) are almost exactly superimposable.

Studies on the Mercapturic Acid Synthesis in Animals. XI. On the Intermediary Metabolism of Benzyl Chloride in Rabbits and Rats. BY JAKOB A. STEKOL. *From the Department of Chemistry, Fordham University, New York*

We have shown previously that benzyl chloride, when administered to dogs, rabbits, and rats, yields in the urine N-acetyl-S-benzylcysteine (benzylmercapturic acid). We now injected benzyl alcohol and benzaldehyde into rabbits and rats and isolated from the urine of these animals hippuric acid. The results indicate that neither benzyl alcohol nor benzaldehyde can be considered as an intermediate substance which is formed from benzyl chloride *in vivo*, and that benzyl chloride probably combines directly with the —SH group of some substance in the animal body and after undergoing further metabolic changes is finally excreted in the urine as benzylmercapturic acid. This substance with which benzyl chloride combines *in vivo* is certainly not homocysteine, as further studies indicated. S-Benzylhomocysteine, when fed to rabbits and rats, yielded in the urine N-acetyl-S-benzylhomocysteine and not benzylmercapturic acid.

Further implications of these results with reference to the synthesis of cysteine and homocysteine *in vivo* and to the mechanism of sensitization of animals to benzyl chloride will be discussed.

The Fate of Guanylic and Adenylic Acids in the Dog. BY FRANK A. V. SULLIVAN AND LEOPOLD R. CERECEDO. *From the Department of Chemistry, Fordham University, New York*

Guanylic and adenylic acids, obtained from yeast nucleic acid, were fed in small amounts (2.5 to 3.0 gm.) to adult dogs maintained on a diet of known composition. The following urinary constituents were determined in 24 hour samples of urine before and after feeding the nucleotides: total nitrogen, urea, creatinine, allantoin, ammonia, inorganic sulfate, and phosphate.

Our observations show that following the feeding of guanylic acid from 44 to 58 per cent of the nitrogen present in the nucleotide is excreted in the form of allantoin. In the case of adenylic acid it was found that from 28 to 43 per cent of the nitrogen was eliminated in the form of allantoin.

Recent workers claim to have observed a rise in the creatinine output of dogs after the administration of yeast nucleic acid. Under the conditions of our experiments, neither guanylic nor adenylic acid caused an increase in the amount of creatinine excreted.

A New and Highly Specific Test for Creatinine. BY M. X. SULLIVAN AND FILADELFO IRREVERRE. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

In a study of creatine and creatinine in various pathological conditions, there was need of a test which would be given by creatinine and not by glycocyamidine or simple hydantoins. In a comparison of the Jaffe test and the 3,5-dinitrobenzoic acid test of Benedict and Behre* it was found that the Benedict and Behre procedure, while more specific in general than the Jaffe test, nevertheless like the Jaffe responded positively with glycocyamidine, simple hydantoins, and certain creatinine derivatives. Accordingly, a new test was devised which has a very high order of specificity for creatinine. This test depends on the reaction of creatinine with 1,4-naphthoquinone-2-potassium sulfonate. Dilute solutions of these compounds mixed and made alkaline make a dye which on acidification gives a brilliant carmine-colored complex extractable by methylene chloride. The reaction is very sensitive. Of a large number of compounds tested the only interference so far met with is with indole. This can be eliminated. The reaction is negative with hydantoins, glycocyamidine, alacreatinine, methyl creatinine, etc. The test is being applied in various biochemical fields.

---

* Benedict, S. R., and Behre, J. A., *J. Biol. Chem.*, **114**, 515 (1936).

The Hemoglobin-Methemoglobin System. The Effect of Urea on the Oxidation-Reduction Potentials. By JOHN FULLER TAYLOR.* *From the Department of Biological Chemistry, Harvard Medical School, Boston*

A stable electrode potential is established only slowly in a mixture of hemoglobin and methemoglobin. It has been found that the addition of a small amount of an electromotively active mediator accelerates the attainment of a reproducible potential, characteristic of the hemoglobin system. Using this technique, we have titrated horse hemoglobin at 30°, between pH 5 and 9. If the mid-point potential, E'_0, of the titration curves, is plotted against pH, the curve is described by the equation

$$E'_0 = +0.168 + 0.0601 \log \frac{[\mathrm{H}^+]}{(2.24 \times 10^{-7}) + [\mathrm{H}^+]} \tag{1}$$

It has been found that the equation

$$E_h = E'_0 + \frac{0.0601}{n} \log \frac{[\mathrm{Ox}]}{[\mathrm{Red}]} \tag{2}$$

does not describe the titration curve of the hemoglobin-methemoglobin system in aqueous salt solution and at constant pH. In passing from hemoglobin to methemoglobin, the experimental points describe a curve which varies progressively from $n = 1$ to $n = 2$. When hemoglobin is titrated in the presence of concentrated urea, the titration curve is described by Equation 2 when $n = 2$.

The Effect of Desoxycorticosterone Acetate (Synthetic Adrenal Cortical Hormone) on the Volume and Electrolyte Content of the Blood Plasma in Patients with Addison's Disease.* By GEORGE W. THORN AND R. PALMER HOWARD. *From the Chemical Division of the Medical Clinic, Johns Hopkins University and Hospital, Baltimore*

The effect of subcutaneous or intramuscular injections of a solution of desoxycorticosterone acetate in sesame oil has been observed in eight patients with Addison's disease. During the period of study the patients were maintained on a constant fluid intake and a diet of constant mineral content. Plasma volume (Gregersen and Gibson†) and serum concentration of electrolytes were determined at appropriate intervals throughout the study.

---

* National Research Council Fellow in Medicine, 1937-38.

* Aided by a grant from the Committee on Research in Endocrinology, National Research Council.

Treatment with desoxycorticosterone acetate resulted in an increase in plasma volume (350 to 1800 cc.) in all of the patients. Withdrawal of treatment resulted in a decrease in plasma volume, while resumption of treatment restored the volume to its previous level. In some patients no immediate increase in the serum concentration of sodium and chloride was observed during the 1st week of treatment. However, the increase in plasma volume which occurred at this time resulted in a considerable rise in the total plasma content of these ions. Continued treatment with desoxycorticosterone acetate restored the serum concentration of sodium and chloride to normal in all of the patients. In those patients in whom the serum level of potassium was previously elevated, treatment with the hormone was followed by a decrease in potassium concentration.

Digestion and Absorption in a Case of Regional Enteritis with Three Feet of Small Intestine.

By W. R. Todd, John R. Montague, Marlowe Dittebrandt, and Edward S. West. *From the Department of Biochemistry, University of Oregon Medical School, Portland*

The study included three periods on a patient operated on for multiple regional enteritis. The first period of 7 days in June, 1935, gave the following results: food intake, carbohydrate 1851 gm., protein 932 gm., fat 1181 gm., calcium 18.47 gm., phosphorus 15.33 gm., and 210 minims of viosterol; percentages of utilization, carbohydrate 99, protein 76, fat 55; 3.65 gm. of calcium and 2.25 gm. of phosphorus were retained. The following results for the second period of 9 days in March, 1938, were obtained: food intake, carbohydrate 2973 gm., protein 1225 gm., fat 1796 gm., calcium 28.4 gm., phosphorus 20.9 gm., and 270 minims of viosterol; percentages of food utilization, carbohydrate 99, protein 75, fat 38; negative balances of 2.08 gm. of calcium and 1.24 gm. of phosphorus were observed. A third period of 7 days on a high carbohydrate, low fat diet was run in April, 1938. The results were as follows: food intake, carbohydrate 3566 gm., protein 1139 gm., fat 679 gm., calcium 21.6 gm., phosphorus 17.6 gm., and 210 minims of viosterol; percentages of utilization, carbohydrate 99, protein 69, and fat 5.5; negative balances of 0.78 gm. of calcium and 0.20 gm. of phosphorus were found. Fecal fat contained about 80 per cent fatty acids in all periods. The per cent of calories lost in the three periods respectively was 26, 35, and 29.

---

† Gregersen, M. I., and Gibson, J. G., Jr., *Am. J. Physiol.*, **120**, 494 (1937).

The excellent utilization of carbohydrate and the marked decrease in fat utilization are striking. Calcium and phosphorus utilization was improved by a high carbohydrate, low fat diet.

Oxidative Reactions of Methionine, Cysteine, and Cystine. By Gerrit Toennies and Thomas P. Callan. *From the Lankenau Hospital Research Institute, Philadelphia*

Some new observations on the differential behavior of the three compounds with hydrogen peroxide are presented. It has been known that cysteine is oxidized by hydrogen peroxide to cystine, that this reaction is catalyzed by cupric ion, that its velocity *de*creases with *in*creasing acidity, and that the oxidation proceeds beyond the cystine stage, leading to cysteic acid. Recently* it has been found that an excess of hydrogen peroxide oxidizes methionine to the sulfoxide stage. The present work shows that the velocity of this oxidation *in*creases with *in*creasing acidity and that it is not influenced by cupric ion. The same, increasing speed with increasing acidity and absence of cupric catalysis, was found to be true for the hydrogen peroxide oxidation of cystine. The velocities of all three oxidations differ widely, and in an acid medium that of methionine is by far the most rapid one. In the presence of a 20 per cent excess of hydrogen peroxide the oxidation of methionine (0.16 M) in 2 M aqueous $HClO_4$ is complete in 10 minutes. Under the same conditions (2 M $HClO_4$, $1.2H_2O_2:1S$, 10 minutes) oxidation of cysteine to cystine amounts to less than 3 per cent, while oxidation of cystine is negligible. Methionine is also oxidized by acid iodine, but, contrary to the well known behavior of cysteine and cystine and its own behavior with hydrogen peroxide, at a very slow rate.

---

* Toennies, G., *Science*, 88, 545 (1938).

The study is being continued with a view to possible applications in protein analysis.

The Hormone Content of the Anterior Pituitary of the Sperm Whale.* By SIBYLLE TOLKSDORF AND H. JENSEN. *From the Biochemistry Laboratory, Squibb Institute for Medical Research, New Brunswick*

The amount of follicle-stimulating hormone and interstitial cell-stimulating hormone (luteinizing hormone) present in a 40 per cent alcohol extract from desiccated anterior pituitaries of the sperm whale was determined quantitatively. The interstitial cell-stimulating hormone content was found to be very low, which is probably due to partial destruction by autolysis. The ratio of follicle-stimulating hormone to interstitial cell-stimulating hormone was compared with that found in similar material obtained from fresh and autolyzed anterior beef and whole sheep pituitaries.

The low interstitial cell-stimulating hormone (luteinizing hormone) content in the anterior whale pituitary runs parallel with a low antagonistic effect. This observation confirms previous studies regarding the identity of the luteinizing hormone, interstitial cell-stimulating hormone, and antagonist factors. The relationship between the thyrotropic and luteinizing effects was studied and the significance of these findings will be discussed.

Sterol Metabolism in the White Rat. By CARLETON R. TREADWELL AND H. C. ECKSTEIN. *From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*

Sterol metabolism was studied in white rats on high fat (28 per cent) and low fat (6 per cent) diets, adequate in caloric value, protein, inorganic salts, and vitamins. The sterol contents of the whole blood, serum, livers, or remaining tissues were no greater on the high fat diet than on the low fat diet. Sterol balances in the females were determined during the various phases of the reproductive cycle; *i.e.*, during pregestation, gestation, lactation, and postlactation. Negative sterol balances (difference between fecal and dietary sterols) were greater in males than in females. The negative balances (male and female) were larger on the high fat diet than on the low one. The neutral fat and phospholipid contents of the livers were also determined.

---

* The material was kindly supplied by Dr. E. M. K. Geiling, Department of Pharmacology, the University of Chicago.

The Inheritance of High Uric Acid Excretion in Dogs. By Harry C. Trimble and C. E. Keeler. *From the Biochemical Laboratory and the Howe Laboratory of Ophthalmology, Harvard Medical School, Boston*

Inheritable characteristics which are susceptible to chemical measurement are relatively scarce. One such character is "high" or "low" uric acid excretion in dogs. Animals of the Dalmatian breed fed a purine-free diet excrete high quantities of uric acid (28 or more mg. per kilo per day), while collies excrete low quantities (less than 10 mg. of uric acid per kilo of body weight per day). First generation Dalmatian-collie hybrids all excreted less than 10 mg. of uric acid per kilo per day. Second generation hybrids (both direct cross and back cross) fell into two groups with respect to uric acid excretion. One group eliminated more than 28 mg. per kilo per day; the other group less than 10 mg. per kilo per day. Thus uric acid excretion was inherited either as a "high" or a "low" character *with no overlapping of classes and with no intermediate grades.* Genetically, it is a simple recessive Mendelian unit character. It is not sex-linked. A single pair of genes determines the character, which is independent of those determining the spotting. Altogether, the uric acid excretions of 78 animals (pure bred and hybrids) have been observed.

The Oxidation-Reduction Potential Requirements of a Non-Spore-Forming Obligate Anaerobe. By Birgit Vennesland. *From the Department of Biochemistry, the University of Chicago, Chicago*

A determination has been made of the apparent oxidation-reduction potential of the medium which just limits the growth of a strain of *Bacteroides vulgatus*, a non-spore-forming, obligate anaerobe. The organisms were inoculated into glucose nutrient broth contained in vessels provided with shiny platinum electrodes to measure the oxidation-reduction potential, and with glass electrodes to measure pH. Potentials were maintained at definite levels by controlling the composition of a nitrogen-air mixture which was bubbled through the culture. A small amount of CO_2 was found to be essential for growth of the organism. The CO_2 was furnished with the gas mixture, which was passed through a saturated $NaHCO_3$ solution before being led into the culture vessel. Potentials of +0.150 volt ±0.010 at pH 6.6 completely inhibited growth; between +0.100 and 0.150 volt there was partial inhibition, whereas below 0.100 volt there was uninhibited growth. Growth was measured by increase in turbidity and was always accompanied by the initiation of acid production. A greater proportion of oxygen was necessary to maintain the potential level at a relatively high positive value when growth was rapid than when growth was slow or when the medium was sterile. Fresh cultures of the anaerobe on glucose brain broth medium showed a relatively high rate of oxygen consumption which decreased rapidly with time. The findings are interpreted by postulating the existence in the anaerobic system of a necessary enzyme which is oxidatively inactivated by oxygen.

Excretion of Sodium Pregnanediol Glucuronidate in the Adreno-Genital Syndrome. By ELEANOR HILL VENNING, PAUL G. WEIL, AND J. S. L. BROWNE. *From the McGill University Clinic, Royal Victoria Hospital, Montreal, and the Department of Surgery, Johns Hopkins Hospital, Baltimore*

The excretion of sodium pregnanediol glucuronidate is reported in two cases of adreno-genital syndrome. The first was a case of adrenal cortical carcinoma in a woman aged 29 years with symptoms of virilism. The patient excreted 12 mg. of sodium pregnanediol glucuronidate daily over a period of 7 days prior to the removal of the right adrenal containing the tumor. Following operation sodium pregnanediol glucuronidate disappeared from the urine. No corpus luteum could be demonstrated in the ovary at autopsy.

The second was a case of adrenal hyperplasia associated with virilism in a woman aged 25 years, who had never menstruated. She excreted 20 to 30 mg. of sodium pregnanediol glucuronidate daily over an observation period of 2 weeks. At exploratory laparotomy, both ovaries were small and showed no evidence of corpus luteum formation; the left adrenal was enlarged.

The relation of this finding to the evaluation of the significance of pregnanediol excretion is discussed.

The Ability of Homocystine Plus Choline to Support Growth of the White Rat on a Methionine-Free Diet.

By Vincent du Vigneaud, Joseph P. Chandler, A. W. Moyer, and Dorothy M. Keppel. *From the Department of Biochemistry, Cornell University Medical College, New York City*

While investigating the ability of homocystine to support growth of animals on a methionine-free diet, we have found that in the presence of choline homocystine is capable of replacing methionine. Apparently in the absence of choline or some related substance the body is unable to methylate homocysteine.

The diet employed was based on Rose's amino acid diet but in place of tikitiki and milk vitamin concentrate, the diet was supplemented with thiamine chloride, riboflavin, nicotinic acid, and ryzamin B. With homocystine or homocysteine in place of methionine the rats failed to grow, but when choline in addition to homocystine or homocysteine was administered, the animals immediately responded with growth as good as that obtained with methionine. Experiments are now under way to test the effect of other methylated compounds as well as substances related to choline.

These observations may explain the efficacy of methionine and the failure of homocystine to decrease fatty infiltration of the liver resulting from high fat, low choline diets.

It is hoped that evidence may be obtained to show whether the methylation of homocysteine in the presence of choline is a direct reaction and, further, whether the methyl group attached to the sulfur of methionine may be the source of the methyl groups in choline. The methionine-homocystine-choline relationship may be significant, not only to sulfur and fat metabolism, but also to other problems of methylation in the animal organism.

Nature of the Abnormal Pigment in the Blood of Individuals Receiving Sulfanilamide Treatment. BY WILLIAM B. WENDEL AND NANNETTE M. WENDEL. *From the Department of Chemistry, University of Tennessee, College of Medicine, Memphis*

The blood of most individuals showing cyanosis from sulfanilamide treatment contains methemoglobin. In many instances the cyanosis is readily explicable on this basis. In some, however, the cause is not so obvious. It has been suggested that aniline black (Marshall and Walzl) or a purple dye (Ottenberg and Fox) may be responsible for the cyanosis as well as the characteristically dark color of the blood of these patients.

Spectrophotometric and gasometric measurements which we have made indicate that methemoglobin is the principal if not the sole cause of the abnormal color of blood from these cyanotic patients. When the visible absorption spectrum of the blood of these individuals is measured and corrected for the oxyhemoglobin content, the residual absorption spectrum corresponds closely, qualitatively and quantitatively, to that of methemoglobin.

The methemoglobin contained in the erythrocytes of individuals receiving sulfanilamide is more stable than that formed by the addition of nitrite to human blood. Experiments will be reported which indicate that this stability is more apparent than real. There is evidence that blood of these patients contains a store of some substance, probably a derivative of sulfanilamide, which continues to form methemoglobin after the blood is drawn. This continued formation of methemoglobin usually just balances the normal spontaneous reduction of methemoglobin, causing the methemoglobin concentration to remain essentially constant for several hours.

Some Properties of Prolactin. BY ABRAHAM WHITE AND ROY W. BONSNES. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The isolation* of a crystalline protein with marked crop-proliferating activity has led to a study of some of the chemical, physical, and physiological properties of this protein. The crystalline nature of the material is indicated by its microscopic appearance and from its x-ray diffraction pattern. The relatively constant physiological activity of the crystalline substance after several recrystallizations suggests that the crystalline protein is identical with the hormone. The chemical individuality is further established by experiments with the cataphoresis apparatus of Tiselius. The ultraviolet absorption spectrum of prolactin has been studied in detail by Dr. G. I. Lavin of The Rockefeller Institute.

---

* White, A., Catchpole, H. R., and Long, C. N. H., *Science*, **86**, 82 (1937).

The elemental analysis of the crystalline substance is typically that of a protein; no phosphorus is present. 0.5 per cent solutions yield precipitates when tested with the common protein precipitants. The ninhydrin, biuret, Hopkins-Cole, and Millon's tests are positive. The test for labile sulfur is strongly positive. Digestion with pepsin or hydrolysis with boiling 20 per cent hydrochloric acid results in a destruction of the physiological activity; destruction of the hormone in solution is most rapid by boiling at pH 13. Solutions of prolactin in 0.1 N sodium hydroxide gradually lose their physiological activity on standing at room temperature.

The crystalline protein appears to be a physiological entity. Assays for growth-promoting, thyrotropic, "diabetogenic," adrenotropic, and gonadotropic activities have all yielded negative results with the methods of assay employed.

Effect of Oral Administration of Methylcholanthrene and of Benzpyrene on the Growth of the Rat.

By JULIUS WHITE AND ABRAHAM WHITE. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The growth-inhibiting action of methylcholanthrene and of benzpyrene has been demonstrated on young, growing rats by the incorporation of suitable quantities of either of these hydrocarbons into a relatively low protein basal diet. The addition of either methylcholanthrene (70 mg.) or benzpyrene (90 mg.) to each 100 gm. of the basal diet results in a change of the daily growth rate from an average of 1.4 gm. on the basal diet alone to an average of 0.3 gm. The addition of either *l*-cystine (400 mg.), *dl*-methionine (500 mg.), or cystine disulfoxide (500 mg.) to each 100 gm. of the basal diet containing the methylcholanthrene results in a stimulation of growth with a subsequent daily weight increment approximating that observed in rats ingesting the basal diet alone. Moreover, a similar growth-stimulating effect is observed following the subcutaneous injection of glutathione (55 mg. daily) into rats ingesting the methylcholanthrene-containing basal diet. On the other hand, supplements of either taurine, glycine, or sodium sulfate did not stimulate growth when these substances were added to the methylcholanthrene-containing diet. The effects of each of the listed supplements on the growth of animals stunted by means of benzpyrene is being investigated. In addition, efforts are being directed toward determining the metabolic fate of ingested carcinogens.

Blood Concentration in Morphine Addicts. By Edwin G. Williams. *From the Laboratories of the United States Public Health Service Hospital, Lexington, Kentucky*

The effect of maintained morphine addiction with strong physical dependence on the concentration of the blood was studied in man by obtaining data on 136 samples of blood from eighteen well controlled addicts. These data were compared with those obtained on 189 blood samples from post-addicts living in a well controlled institutional environment. A smaller number of blood samples was studied through the withdrawal and early postwithdrawal periods, and on 55 normal male employees of ages comparable to those of the patients. Values were determined for cell volume, specific gravity of whole blood and plasma, and water content of whole blood and plasma. It was found that addiction with strong physical dependence is associated with blood hydration. During withdrawal there is a temporary decrease in hydration, but the concentration does not reach the normal level. Post-addicts have normal values for cell volume, specific gravity of plasma, and water content of whole blood.

Fat Excretion and the Fecal Lipid Distribution of Average, Healthy Children. By Harold H. Williams, Marion L. Shepherd, and Elizabeth E. Endicott. *From the Research Laboratory of the Children's Fund of Michigan in Cooperation with the Methodist Children's Home Society, Detroit*

During a metabolic study of ten average, healthy, preadolescent children over 8 consecutive months under controlled conditions of environment and diet, data were procured upon the excretion of fat, the partition of the fecal lipids, and the absorption of fat upon varying intakes. The coefficients of apparent digestibility indicate a relatively complete absorption of the food fat, averaging over 97 per cent during a period in which the intakes were increased from 57 to 100 gm. daily, approximately. The distribution of the lipids in the average daily excretion of fat (approximately 2 gm. per child) was as follows: soaps 0.27 gm., free fatty acids 0.83 gm., neutral fat 0.75 gm., unsaponifiable 0.10 gm. The variations in the retentions of the different children were much wider than those occurring in the same individual, indicating that fat excretion is characteristic of the individual.

During a large part of the experimental period two of the children received a doubled milk intake. The stools of these children during the period of increased milk intake averaged 15.73 per cent fat; for the children on the lower milk content diet the average was 11.88 per cent. The total excretion of fat increased approximately one-third (average 3.14 gm. daily) and had the following composition: soaps 0.96 gm., free fatty acids 1.12 gm., neutral fat 0.87 gm., and unsaponifiable 0.17 gm. The greatest increase was in the soap constituent, apparently as a result of the increased calcium intake. The soaps and free fatty acids did not vary inversely in the feces, demonstrating that a loss of fat occurred with the increased mineral intake. The small increases in the free fatty acids and neutral fats, accompanying the large increases in fecal soaps, may indicate that they derive, primarily, from the intestinal bacteria.

The Distribution in the Body of Magnesium Salts Injected Intravenously. BY ALEXANDER W. WINKLER, PAUL K. SMITH, AND BERNARD M. SCHWARTZ. *From the Departments of Internal Medicine and of Pharmacology and Toxicology, Yale University School of Medicine, New Haven*

Isotonic solutions of magnesium sulfate were injected intravenously into dogs, and the concentrations of both ions were determined in serum and in urine at intervals after the injection. The apparent volume of distribution of each ion was calculated by the formula

$$\text{Apparent volume of distribution} = \frac{\text{Amount injected minus amount excreted in urine}}{\text{Rise in concentration in serum water}}$$

It was found that the apparent volume of distribution of magnesium was approximately equal to that of sulfate during the first 2 or 3 hours after injection. Since there is abundant evidence that sulfate, like sodium and chloride, is confined to the extracellular fluid, these experiments indicate that magnesium likewise initially distributes itself through the extracellular fluid only. This distribution contrasts with that of potassium, which when studied by the same technique appears to enter the intracellular water as well. After 2 or 3 hours the volume of distribution of magnesium frequently appears to increase somewhat. The eventual fate of the magnesium is now under investigation.

Concentration of a Growth Factor for Clostridium butylicum.

By D. W. Woolley and L. E. McDaniel. *From the Departments of Biochemistry and Agricultural Bacteriology, University of Wisconsin, Madison*

An essential substance for the growth of *Clostridium butylicum* has been detected in a wide variety of natural products. The best source for concentration procedures was a commercial extract of cereal grains known as vitab. Growth was measured by quantitative determination of turbidity with an Evelyn photoelectric colorimeter. A basal medium containing glucose, asparagine, and inorganic salts was developed in which negligible growth occurred. When preparations of the growth factor were added, however, luxuriant growth was obtained.

The active substance could be obtained from an acid aqueous solution of vitab by continuous ether extraction for long periods. It was not extracted by ether from neutral solution. A more convenient method was to shake out the aqueous solution with butanol, then take the growth factor from the butanol with sodium hydroxide solution, and finally shake out from the acidified extract with ethyl acetate. Adsorption on norit and elution with alcohol yielded a concentrate which gave maximum stimulation when 0.1 to 0.5 microgram per cc. was added, and which could be readily detected after the addition of 0.001 microgram per cc.

The distribution between water at various pH values and ether has indicated that the growth factor is an acid. Other acidic growth factors could not replace it. From a study of its stability to various chemical treatments it was concluded that it was an easily oxidizable, alkali-stable, saturated acid of very low volatility.

Ultracentrifugal and Electrophoretic Analysis of the Native Proteins in Egg White. By E. Gordon Young. *From the Department of Biochemistry, Dalhousie University, Halifax, Canada*

An attempt has been made to determine the native state of the various proteins in the white of hen's eggs by the use of the ultracentrifuge. Centrifugation at 60,000 R. P. M. for 5 hours of natural egg white and of that diluted once to several times with 1 per cent sodium chloride resulted in a single sedimenting boundary by the diagonal schlieren method.

After dialysis of natural egg white in 0.1 μ phosphate buffer at pH 7.2 the protein solution was electrolyzed in a Tiselius electrophoresis apparatus. Five or six separate migrating boundaries were demonstrable with monochromatic light of $\lambda = 589$ mμ and a total protein concentration of 5.6 per cent or less.

A careful fractionation of egg white for mucoproteins yielded (1) ovomucoid which was monodisperse in the centrifuge but exhibited two or more boundaries by electrophoresis, and (2) ovomucin which showed two components by centrifugation, yet only one boundary by electrophoresis. Present methods of separation are therefore inadequate for these proteins.

The Biological Synthesis of Hippuric Acid in Vitro. By Henry Borsook and Jacob Dubnoff. *From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena*

A micromethod for measuring very small quantities of hippuric acid (0.1 cc. of 0.0001 M) was devised. With this method and the tissue slice technique, liver and kidney tissues of the rat, rabbit, and guinea pig were found capable of synthesizing hippuric acid from benzoic acid and glycine. The function was absent from the liver of the dog, though present in the kidney of this animal.

Thermodynamic data indicate that under our experimental conditions at equilibrium hippuric acid is 95 to 99 per cent hydrolyzed. Under the same conditions with tissue slices approximately 50 per cent of the benzoic acid is converted into hippuric acid. There is, therefore, a gain in free energy which is supplied by an energy-yielding reaction.

No synthesis was observed with ground up tissues, or when the organs had been removed from the animal for more than 3 hours. The synthesis was evidently associated with intact intracellular structure.

The synthesis proceeds about twice as fast in the liver as in the kidney. It is a slow reaction, about one-thirtieth the rate of the synthesis of urea.

The synthesis proceeds most rapidly when the amino acid used is glycine. With serine and hydroxyproline a substance is formed which behaves like hippuric acid in the usual analytical procedure for hippuric acid. All other amino acids, including threonine, were negative. We are at present engaged in the isolation and identification of the compounds formed by benzoic acid with glycine, serine, and hydroxyproline.